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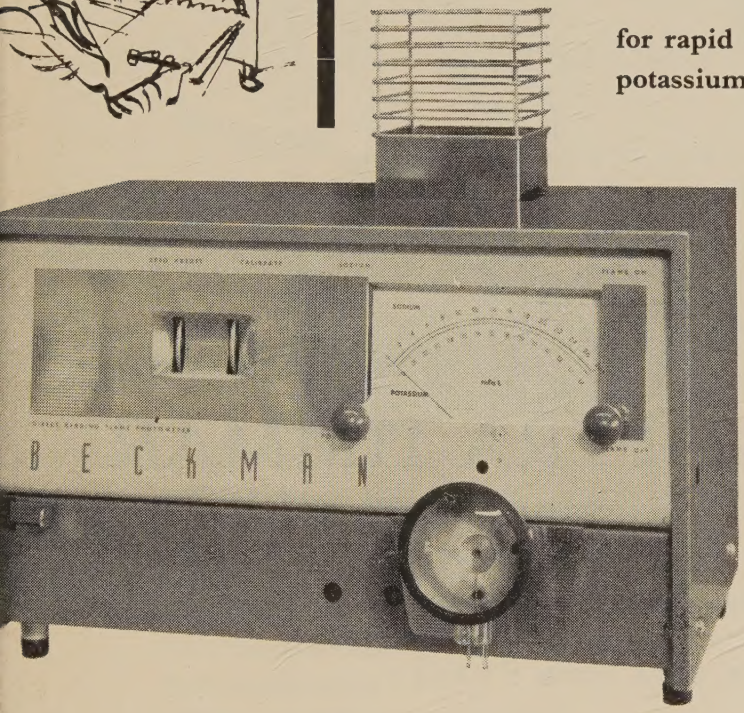

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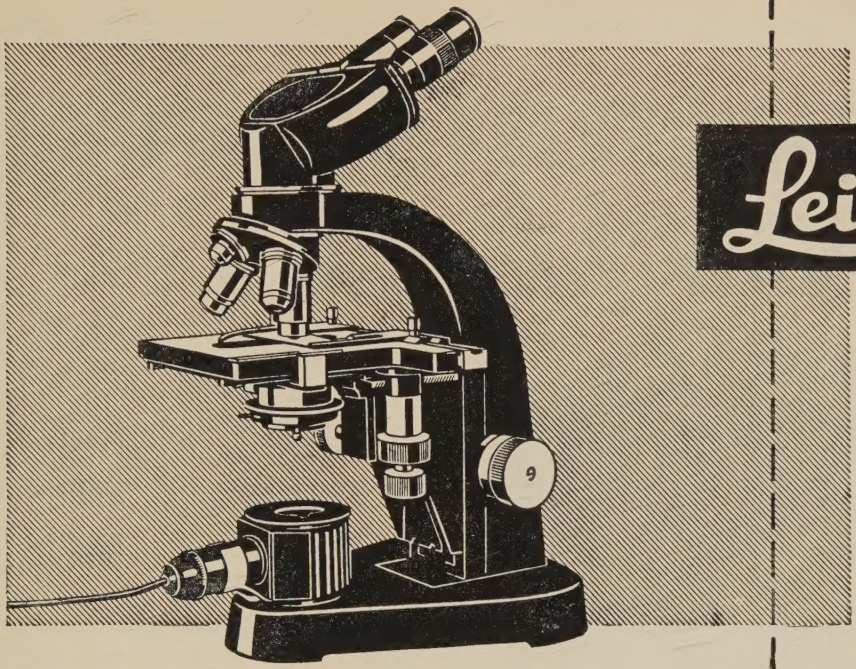

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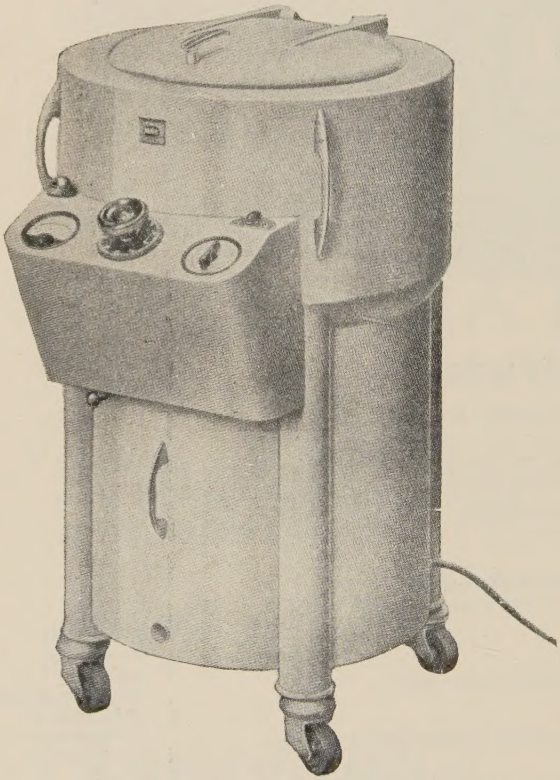

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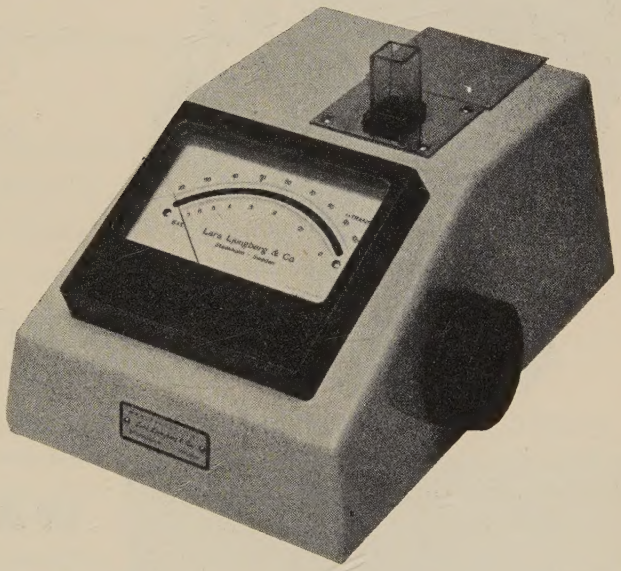

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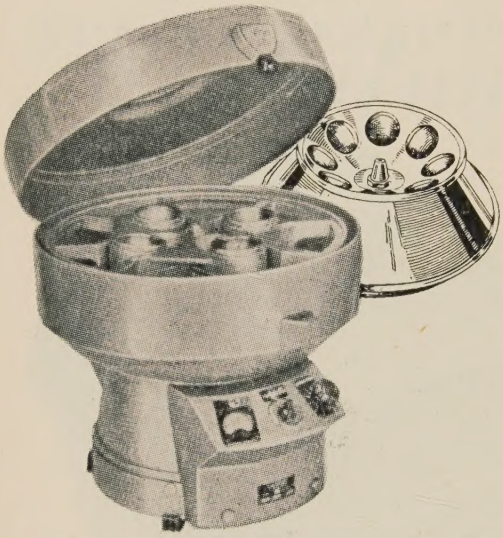

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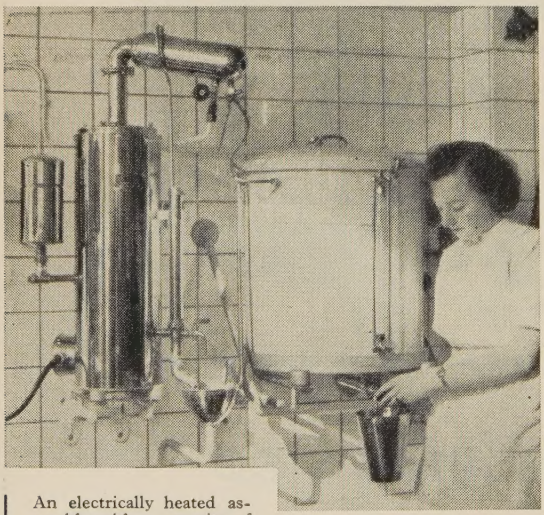

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MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS IN URINE WITH REFERENCE TO THE INFLUENCE OF HOMOGENTISIC ACID

By S. CHR. SOMMERFELT*

From The Clinical Laboratory, Drammen Hospital, Drammen, Norway

(Received for publication March 5, 1956)

Microbiological methods have been used extensively for the determination of amino acids. They have the advantage over chemical methods in that the same principle of analysis is applied to all amino acids. They are simple, although quite time-consuming, and very small quantities can be estimated with a reasonable degree of accuracy.

The basis of all the methods is the fact that a microorganism requires a number of different amino acids in order to obtain maximum growth. If one of these is omitted from the medium, growth will fail, but if it is restored, the amount of growth that occurs will be in proportion to the amount of amino acid supplied. Thus by adding known amounts of the amino acid which is lacking to one series of tubes and measuring the bacterial growth, *e. g.* by titration of the acid formed by the bacteria (usually belonging to the family *Lactobacteriaceae)*, a standard reference curve is obtained. To another series are added known amounts of the sample to be analysed. The amount of amino acid present in each of these sample tubes is found directly by reading the titration value on the standard curve.

An ordinary pipetting technique is generally used with an arithmetical or a geometrical dilution principle. A special "drop method" has been recommended (Seligson, Torghele & Anderson, 1950), when only small amounts of material are available. However, Ågren (1949 b, 1951) has worked out a more accurate micro-pipetting technique for this purpose, which requires only minute amounts.

The method described here is based upon a macro-method of Ågren (1948, 1949 a) with some minor modifications.

* With the assistance of Miss H. A. Simmonds, M.Sc. and Miss B. L. Stewart, M.Sc., New Zealand.

METHOD

Apparatus

Calibrated *pipettes.*
Calibrated *volumetric flasks.*
Pyrex *Erlenmeyer flasks,* 100 ml.
Pyrex *test tubes,* 13 mm × 100 mm.
Metal racks for approximately 50 tubes.
Autoclave (15 lbs./sq.in. — 121° C.)
Incubator (37° C).

Reagents

Sulphuric acid for hydrolysis. 1 vol.conc. H_2SO_4 + 1 vol. distilled water.

Sulphuric acid for pH correction. Approx. 0.6 n.

Sodium hyroxide for pH correction. Approx. 0.75 n.

Table I. *Standard amino acid solutions.*

Amino acid	Concentration of amino acid in standard solution. mg per 100 ml.	Amount of l-amino acid in 0.5 ml of the standard solution μg
dl-aspartic acid ..	1.0	2.5
l-cystine	0.4	2.0
l-glutamic acid ..	5.0	25.0
glycine	0.8	4.0
l-histidine	0.5	2.5
dl-isoleucine	1.6	4.0
dl-leucine	1.6	4.0
l-lysine 2 HCl	1.5	5.0
dl-methionine	0.5	1.25
dl-phenylalanine ..	0.5	1.25
l-proline	0.8	4.0
dl-serine	2.0	5.0
dl-threonine	2.0	5.0
dl-tryptophan	1.0	2.5
l-tyrosine	0.5	2.5
dl-valine	1.6	4.0

Sodium hydroxide for neutralisation. Approx. 12 n.

Sodium hydroxide for titration. Approx. 0.02 n.

Bromothymol blue, indicator. 0.02 per cent in 45 per cent ethanol.

Sodium chloride solution. 0.9 per cent, sterile.

Glass redistilled water.

Standard amino acid solutions. These were prepared from l or dl forms of the acids (La Roche)

Table II. *Composition of basal media.*

		Per 100 ml basal medium
Amino acids (Sum 688.8 mg):		
dl-alanine		40.0 mg
l-arginine HCl		48.4 »
dl-aspartic acid		80.0 »
l-asparagine		80.0 »
l-cysteine HCl		13.0 »
l-glutamic acid		60.0 »
glycine		20.0 »
l-histidine		10.0 »
dl-isoleucine		50.0 »
dl-leucine		50.0 »
l-lysine 2 HCl		50.0 »
dl-methionine		20.0 »
dl-phenylalanine		20.0 »
l-proline		20.0 »
dl-serine		10.0 »
dl-threonine		40.0 »
dl-tryptophan		8.0 »
l-tyrosine		20.0 »
dl-valine		50.0 »
Glucose		5,000.0 »
Sodium acetate · 3 H_2O		4,000.0 »
Ammonium chloride		600.0 »
Vitamin fraction (made up to 100 ml):		
Riboflavine	5.0 mg	
Nicotinic acid	10.0 »	
Calcium dl-pantho-tenate	5.0 »	
Biotine	0.01 »	
Pyridoxamine HCl ..	3.0 »	
Pyridoxine HCl	10.0 »	
Pyridoxal HCl	3.0 »	
Para-amino, benzoic acid	1.0 »	
Thiamine HCl	5.0 »	
Folic acid	0.1 »	5.0 ml
Xantine (made up to 100 ml)	100.0 mg	1.0 »
Purines (made up to 100 ml):		
Adenine sulphate	110.0 »	
Guanine HCl · 2 H_2O	104.0 »	
Uracil	96.0 »	0.8 »
Salt A (Baumann) (made up to 100 ml):		
K_2HPO_4	6,000.0 mg	
KH_2PO_4	6,000.0 »	1.2 »
Salt B (Baumann) (made up to 100 ml):		
$MgSO_4$ · 7 H_2O	2,000.0 mg	
$FeSO_4$ · 7 H_2O	100.0 »	
NaCl	100.0 »	
$MnSO_4$ · H_2O	57.0 »	1.0 »
Salt B_2 (made up to 100 ml):		
$MnSO_4$ · H_2O	95.0 mg	1.0 »

dissolved in glass redistilled water (tyrosine with the aid of some drops of sodium hydroxide), see Table I. They were kept in a refrigerator at + 4° C and renewed every four weeks.

Basal media (Table II). Initially we made stock solutions of each reagent for the media, in accordance with the original procedure (Ågren, 1952). However, later we found it more convenient and time saving to prepare the amino acid portions of the media in powder form. The results obtained by the two procedures were compared and no significant difference was found.

The medium used for the determination of one amino acid contains all the reagents mentioned in Table II except the amino acid to be determined. All the other amino acids were weighed and thoroughly mixed in a mortar, and the powdered mixture was kept in a dessicator. To make a portion of *e.g.* 200 ml basal medium for tyrosine, (688.8—20.0) × 2 mg of the amino acid powder was transferred to a 200 ml volumetric flasks. The appropriate amounts of the other substances and the stock solutions were added. The volume was made up to 200 ml with glass redistilled water and the pH adjusted to 6.8. The medium was kept at — 20° C.

Bacteria. A strain of *Leuconostoc mesenteroides* P60[1] was employed. It was grown in an agar medium (Table III) and kept in a refrigerator. The strain was subcultured every month.

Two days prior to the experiment the agar culture was incubated at 37° C. After 24 hours a round bottomed centrifuge tube containing broth (Table III) was inoculated with material from the agar culture, and after another 20—24 hours the broth culture was ready for use.

To prepare the appropriate suspension the broth tube was first centrifuged at 3000 revolutions per minute for 20 minutes and the supernatant fluid was decanted. The bacteria were then washed twice with sterile saline, and finally they were suspended in 50—75 ml sterile saline at 37° C, making a bacterial suspension of approximately 100 millions of bacteria per ml.

[1] Obtained from Prof. Ågren, Uppsala, to whom I am greatly indepted.

Table III. *Medium for leuconostic mesenteroides P 60.*

Broth: 1 gm Sodium acetate
1 gm Glucose
0.5 gm Bactopeptone (Difco)
0.1 gm Yeast extract (Difco)
0.5 ml Salt A (see Table II)
0.5 ml Salt B (see Table II)
Glass redistilled water ad 100 ml.

Solid medium:

This had the same composition as the broth with the addition of 1.5 gm agar (Difco) per 100 ml.

Procedure

The determination of free + conjugated amino acid was made after hydrolysing the urine. 0.64 ml of the 50 per cent sulphuric acid was added to a 5 ml sample of urine and autoclaved for 5 hours at 15 lbs./sq.in. (121° C). The hydrolysate was approximately neutralised with several drops of concentrated sodium hydroxide solution and diluted to a urine concentration of $^1/_5$ to $^1/_{40}$, depending upon the amino acid to be determined. The pH was then corrected to 6.8 by means of a dilute sodium hydroxide solution.

The amount of free amino acid was determined in the untreated urine with dilutions varying from $^1/_2$ to $^1/_{40}$ and a pH of 6.8.

For each amino acid determination 0.5, 0.4, 0.3, 0.2 and 0.1 ml of the standard amino acid solution and 0.5, 0.4 and 0.3 ml of each sample dilution were pipetted in duplicate into test tubes. Glass redistilled water was added to bring the volumes up to 0.5 ml. Two tubes with 0.5 ml of water were the zero point of the standard curve. Then 0.5 ml of the appropriate medium was added to each tube and the racks were covered with cloths. This was found to be more convenient than individual cotton wool plugs. The tubes were autoclaved for 10 minutes at 15 lbs pressure and after cooling to room temperature the cloths were removed, and one drop of the freshly prepared saline suspension of *Leuconostoc mesenteroides* was added to each tube. The covers were replaced and the tubes incubated for 65—70 hours at 37° C, which was found to be the optimum incubation time for all amino acids studied here, except for glycine, which

was incubated only 16 hours. The acids produced by the bacteria were then estimated by titration with $^1/_{50}$ N sodium hydroxide solution, using 0.02 per cent bromothymol blue as indicator.

The titration value of each standard tube was plotted against the amount of amino acid in the same tube, and a curve, the standard curve, was drawn. The titration values for the sample tubes were read on this curve, giving directly the amount of amino acid present in each tube. When less than four titration values for each sample fell within the limits of the standard curve, the analysis was repeated with an appropriate dilution of the urine. The concentration of the amino acid in each urine specimen was thus the average of 4—6 determinations. Up to 40 samples were analysed for one amino acid at the same time.

In alkaptonuric urine it is of particular interest to determine the content of phenylalanine and tyrosine, as alkaptonuria is supposed to be a disorder of the intermediary metabolism of these amino acids. The homogentisic acid present in such urine renders the determination a little more difficult than usual because of the darkening in color both during the hydrolysis and during the three days incubating period. This dark color interferes with the judgement of the end point during titration, and we therefore made attempts to remove the homogentisic acid from the urine before analysis. However, the procedures which removed most of the homogentisic acid, *viz.* precipitation with plumbic acetate and absorption with charcoal, also removed a part, and often the greater part, of the phenylalanine and tyrosine. The only practical solution found was to dilute the urine more than usual before analysis.

RESULTS

A disadvantage of the method described is that it gives unsatisfactory results if the amino acid concentration in undiluted urine is low, because some "inhibitory factors" then appear to come into play. Before the analysis is performed it is necessary to dilute the urine at least 1 : 2. The method was found to work in a satisfactory way in unhydrolysed urine with the following amino acids: glycine, histidine, phenylalanine, tryptophan and tyrosine. Some other amino acids could sometimes be determined, but not always, *e. g.* isoleucine, leucine and threonine, depending upon the concentration of the acids in the different urine samples. After hydrolysis of the urines, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine could be determined.

Reproducibility

This was checked by repeated analyses of some urine samples. The analyses were carried out separately from the very beginning. Table IV shows some results for an arbitrary selection of amino acids both with normal and with alkaptonuric urine.

In practical work it is seldom possible to analyse the urine sample as soon as it is voided. As this is a microbiological method it is theoretically unsatisfactory to add antibacterial preservatives to the urine. It is better to freeze the urine and keep it at — 20° C until it can be dealt with. However, it may sometimes be inconveniant or difficult to freeze the urine immediately after voiding, and we therefore made some experiments on the influence of standing at room temperature for some hours before freezing. Urine samples with a high concentration of the amino acid to be determined (obtained by feeding phenylalanine and, another day, tyrosine to a normal person) were left for 10 and 17 hours respectively before freezing. On analysis the results were consistent with those obtained

Table IV. *Examples of reproducibility with microbiological determination of some amino acids in normal and alkaptonuric urine.*

		Glycine µg/ml	Histidine µg/ml	Isoleucine µg/ml	Phenylalanine µg/ml	Threonine µg/ml	Tryptophan µg/ml	Tyrosine µg/ml
Normal urine	Unhydrolysed	130	188		12.5		24	28
		121	185		12.2		19	26
		119			11.5			
	Hydrolysed	258	156	17	18.4	25		69
		234	145	14	17.6	23		68
								67
Alkaptonuric urine	Unhydrolysed				15.0			30
					14.2			28
	Hydrolysed				25.2			77
					23.8			74

Table V. *Possible influence on urine specimens of standing at room temperature for some hours before freezing.*

	Phenylalanine µg/ml	Tyrosine µg/ml
Frozen immediately ...	44.0	148.0
Kept at room temp. for 10 hrs., phenyl., and 17 hrs., ty.	44.4	147.0

from other portions of the same samples which had been frozen immediately after voiding (Table V). However, it would presumably be unsafe to leave an infected urine with vigorous bacterial growth standing for a period of time at room temperature before freezing.

Recovery

These experiments were carried out by adding known amounts of the amino acid to be determined to different urine specimens. The amino acids were added either in solution or as powder in amounts of about 50 per cent of that present in the original samples. This entailed an increase in the dilution of the urine samples. Table VI shows the percentage recoveries which varied from approximately 90 to 110 per cent for phenylalanine and tyrosine, although some of the other amino acids showed greater variation.

Specificity

Previous work indicates that the microbiological determination of amino acids has a high specificity. Therefore we have made no extensive experiments in this connection. However, as will be reported in later articles, there is a striking parallellism between the excretion of phenylalanine and of tyrosine in alkaptonuric urine. In order to exclude the possible influence of tyrosine on the estimation of phenylalanine and *vice versa*, tyrosine was added to an alkapton-

Table VI. *Recoveries with microbiological determination of some amino acids in normal and alkaptonuric urine.*

		Glycine per cent	Isoleucine per cent	Leucine per cent	Phenylalanine per cent	Threonine per cent	Tyrosine per cent	Valine per cent
Normal urine	Unhydrolysed	128		86	104 101 100		106 104 98 91	
	Hydrolysed	115	125 121 106 93	94	103 90	109	107* 101* 101 91	96
Alkaptonuric urine	Unhydrolysed				111 104		108 97 92	
	Hydrolysed				110 96		102 101 89 89	

* Minute amounts added, 0.25 μg per 0.5 ml.

Table VII. *Influence of tyrosine on the estimation of phenylalanine and vice versa.*

	Phenylalanine μg/ml	Tyrosine μg/ml
Untreated sample	12.5	24.8
Addition of 25 μg tyrosine per ml	14.0	
Addition of 10 μg phenylalanine/ml		24.5
Difference	+1.5	—0.3

uric urine sample in an amount sufficient to double the original content of this amino acid. The urine was then analysed for phenylalanine at the same time as the original sample without addition. *Mutatis mutandi* the same was done with the other amino acid. As seen from Table VII the addition had no significant effect on the results.

DISCUSSION

The values reported in Table IV for normal urines seem to be in fairly good agreement with those stated by other authors (Dunn, Camien, Shankman & Block, 1947; Frankl & Dunn, 1947; Woodson, Hier, Solomon & Bergheim, 1947; Yeh, Frankl, Dunn, Parker, Hughes & György, 1947; Eckhardt & Davidson, 1949; Kirsner, Sheffner & Palmer, 1949). However, they are given in micrograms per ml, which is convenient for judging the recoveries, but obviously misleading if one wants to know the "normal values". These must be expressed as mg per 24 hours.

Further, the values for phenylalanine and tyrosine in alkaptonuric urine were also found to be within the normal range.

The reproducibility of results (Table IV nd V) should be satisfactory for a bio-gical method of this kind, as most amino cids show a variation of about 10 per cent. However, for some, particularly those hich are present in low concentrations in he urine, it goes up to 25—30 per cent. In he case of recovery results for the two mino acids which have been subject to by ar the greatest number of analyses, phenyl-lanine and tyrosine, they are within ± 10 er cent of the amount added (Table VI). ome of the others are less satisfactory.

In man there is such a great variation in he amounts of amino acids excreted in the rine, that only a great deviation from the ormal values could be regarded as patho-gical. For this purpose the accuracy of he method will suffice, and it offers better nformation on the quantitative variations han does ordinary paper chromatography.

The presence of homogentisic acid in a rine sample could possibly influence the esults obtained by interference with the acterial growth. Either it might activate he growth, *e. g.* in the estimation of phenyl-lanine and tyrosine the bacteria could make se of this substance instead of the amino cid to be determined. However, this is un-kely, because of the specificity of the bac-eria in their requirements for growth. Or, n the other hand, it might retard the bac-erial growth, by acting as an inhibitory ubstance in one way or another. In order o elucidate these possibilities, homogentisic cid should be added to a normal urine ample and the influence on the results of he amino acid analyses established. We ave not yet performed such experiments, but we feel that our reproducibility and recovery results show that this possible influence of homogentisic acid on bacterial growth can hardly be of any practical size.

SUMMARY

1. A microbiological method, employing *leuconostoc mesenteroides* P 60 for the determination of amino acids in urine is described. In unhydrolysed urine glycine, histidine, phenylalanine, tryptophan and tyrosine could always be determined. After hydrolysis the method is satisfactory also for asparctic acid, cystine, glutamic acid, isoleucine, leucine, lysine, methionine, proline, serine, theorine and valine.

2. The reproducibility of the method is fairly good for most of the amino acids mentioned above.

3. Recovery experiments show a variation of 86 to 128 per cent. More satisfactory results (90 to 110 per cent) were obtained with phenylalanine and tyrosine.

4. There is no significant influence of tyrosine on the estimation of phenylalanine and vice versa.

5. The dark color caused by the homogentisic acid renders the microbiological determination of amino acids in alkaptonuric urines difficult. This is overcome by employing a greater dilution of the urine than that described for normal urines. The reproducibility and recovery with alkaptonuric urine are similar to those obtained with normal urine.

ACKNOWLEDGMENT

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THE EFFECT OF HYDRALAZINE (APRESOLIN) ON KIDNEY FUNCTION AND SODIUM EXCRETION[1]

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The question of the effect of hydralazine n the kidney function has apparently not een fully elucidated. In a preliminary re- ort, Reubi (1950) stated that the renal lood flow was increased both in normal ndividuals and in hypertonics, whereas the ffect on the glomerular filtration rate was nconstant.

In the more recent studies, in which the ffect of a single dose of hydralazine has een examined (Judson & Hollander, 1954; Verkö, Ek, Eliasch, Varnauskas, Holm- ren & Bucht, 1953, and Wilkinson, Back- an & Hecht, 1952), it is apparently agreed nat the renal blood flow increases in all ases in which the blood pressure does not all to shock-level. The same seems to apply o peroral administration (VanderKolk, Pontas & Hoobler, 1954). In these papers, oo, the effect on the glomerular filtration ate is said to vary.

The effect on the sodium excretion was xamined by Werkö (1953), who found a owering of the sodium excretion, and by Judson & Hollander (1954), who demonstrated a rising sodium excretion in patients with heart failure, whereas it remained unchanged in persons with normal hearts.

The results given in the following seem to suggest that the varying effect on kidney function and salt excretion depends on the extent to which the dose of hydralazine administered influences the blood pressure.

TECHNIQUE

The investigations reported here were performed on 6 patients admitted to the department with essential hypertension; heart failure was present in none.

The endogenous creatinine clearance and the PAH-clearance were determined. In addition the urine flow and the sodium excretion were measured. Following 4 control periods, hydralazine was injected intravenously in doses varying from 10 to 20 mg, or from 0.11 to 0.32 mg per kg of body weight. When the hydralazine effect had commenced, after a lag varying from 10 to 30 minutes, 4 or 5 additional clearance periods followed. A catheter was passed into the bladder, and quantitative evacuation was secured by irrigation of the bladder twice with 50 ml of water plus 50 ml of air. The duration of the separate clearance periods was about 10—15 minutes.

The PAH-analyses were performed according to Brun's method (1951). The creatinine-analyses

[1] Aided by a grant from King Christian X's oundation. Hydralazine (Apresolin) was kindly aced at our disposal by CIBA A/S, Copenhagen.

were made according to Bosnez & Taussky's technique (1945). The sodium analyses were performed by flame photometry with Beckman's photometer DU.

RESULTS

Table I gives a complete survey of the results. The experiments are arranged according to decreasing effect on the mean blood pressure. The mean pressure was calculated as the diastolic blood pressure plus one-third of the amplitude. In experiments 1 and 2, a considerable fall in the mean blood pressure was effected. Figures 1 and 2 give the details of these experiments. During the second hydralazine period in Case 1 there was a considerable fall in blood pressure (to 95/75 mm Hg), and the patient became pale and cool, but recovered spon-

Table I.

No.	Sex	Age	Blood pressure mm Hg C.	Blood pressure mm Hg H.	Mean blood pressure mm Hg C.	Mean blood pressure mm Hg H.	Pulse rate C.	Pulse rate H.	Dose of hydralazine total mg	Dose of hydralazine mg/kg	Creatinine-clearance ml per min. C.	Creatinine-clearance ml per min. H.	PAH-clearance ml per min. C.	PAH-clearance ml per min. H.	Filtration fraction C.	Filtration fraction H.	Diuresis ml per min. C.	Diuresis ml per min. H.	Sodium excretion mEq per min C.	Sodium excretion mEq per min H.
1.	M.	57	220/140	(150/105)	167	(120)	72	92	20	0.24	67	37	235	182	28.5	20.3	7.3	1.8	0.93	0.20
2.	F.	68	235/135	(185/105)	168	(133)	116	100	12	0.16	76	71	348	432	21.8	16.4	6.1	1.3	0.45	0.23
3.	F.	68	165/85	150/65	113	94	60	72	20	0.32	38	39	121	206	31.4	18.9	4.0	1.7	0.13	0.12
4.	F.	51	170/100	160/85	122	110	60	84	14	0.23	57	57	330	394	17.3	14.5	1.7	2.2	0.32	0.62
5.	M.	44	180/115	180/105	137	131	68	76	12	0.14	142	142	683	893	20.8	15.9	0.6	0.8	0.16	0.24
6.	F.	59	150/95	150/85	113	107	82	96	10	0.11	72	80	344	420	20.9	19.0	2.7	5.1	0.13	0.24

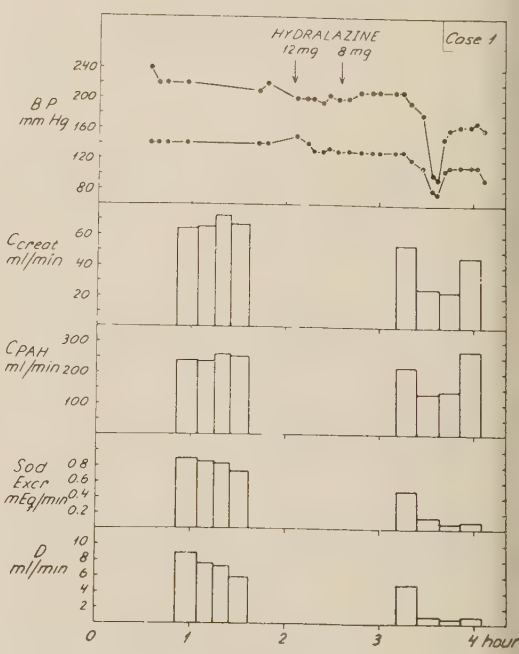

Fig. 1.

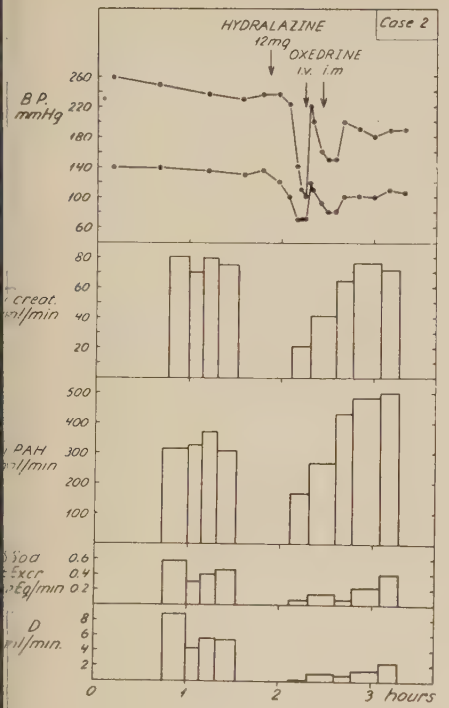

Fig. 2.

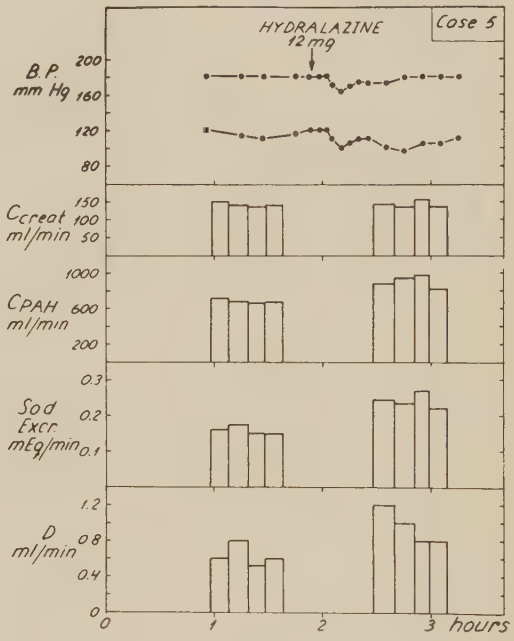

Fig. 3.

neously. In conjunction with the fall in od pressure the creatinine and PAH-clear-nces diminished, and there was a pronounced ll in output of urine and sodium excretion. uring the first hydralazine period in Case the blood pressure fell to 100/70 mm Hg, nd the patient became pale, perspiring and veloped stenocardia. Oxedrine injections ere required twice with an interval of a w minutes; the blood pressure then rose gain. As in the first case there was a fall clearance values, output of urine and odium excretion. In addition it is seen that e output of urine remained low, and that e sodium excretion rose but a little, even though creatinine and PAH-clearances higher than those found during the control periods were obtained after injection of oxedrine.

In Case 3 there was a moderate effect on the mean blood pressure. The PAH-clearance rose, whereas the creatinine clearance and the sodium excretion remained constant.

In Cases 4, 5 and 6 the mean blood pressure fell very moderately. The pulse rate rose by 15 beats per minute on the average. It appears that the creatinine clearance remained unchanged, whereas the PAH-clearance rose. The filtration fraction fell. The output of urine remained fairly unchanged,

whereas the sodium excretion rose in all 3 patients. Fig. 3 illustrates one of these experiments.

DISCUSSION

As will appear, the effect of hydralazine on kidney function and sodium excretion may be highly variable, dependent on the hemodynamic effect obtained.

A drastic lowering of the blood pressure will produce a fall both in the glomerular filtration rate and in the renal blood flow; at the same time the sodium excretion falls. A very moderate effect on the blood pressure will cause the renal blood flow to rise, whereas the filtration rate remains unchanged; at the same time the sodium excretion may rise.

A fall in the filtration fraction was seen in all experiments; this shows that the renal blood flow is either absolute or, in certain experiments, comparatively large in relation to the glomerular filtration rate.

It is apparently common to all hypotensive agents that they produce a fall in the sodium excretion; this applies to hydralazine, too, when the hypotensive effect is pronounced. Hydralazine seems, however, to be in an exceptional position, as this may increase the sodium excretion in the case of moderate effect on the blood pressure.

A sodium retention will always counteract the therapeutical effect of a hypotensive agent on the blood pressure, as shown especially in the case of pentapyrrolidine by Rønnov-Jessen (1955). A sodio-uretic effect produced by hydralazine would therefore be therapeutically valuable. It cannot be said at present whether hydralazine also in therapeutical doses promotes the excretion of sodium.

SUMMARY

Six patients admitted to the department with hypertension received intravenous injections of from 0.11 to 0.22 mg of hydralazine per kg of body weight. The effect on the kidney function and the sodium excretion varied, dependent on the hemodynamic effect obtained.

A drastic lowering of the blood pressure results in a fall both of the glomerular filtration rate and the renal blood flow; at the same time the excretion of sodium falls. A very moderate effect on the blood pressure causes the renal blood flow to increase, whereas the glomerular filtration rate remains unchanged; at the same time the sodium excretion may rise. A fall in the filtration fraction was seen in all experiments.

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PHYSICAL AND BACTERIOLOGICAL INVESTIGATIONS OF AN ACRYLIC FILM INTENDED AS A MATERIAL FOR SURGICAL DRESSINGS[1]

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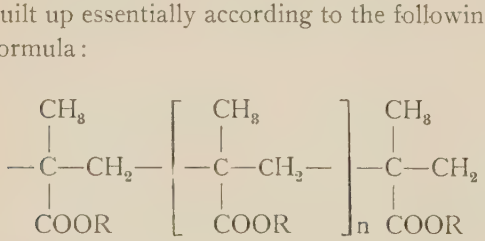
INTRODUCTION

The favorable results obtained with plastic materials in various branches of surgery prompted many workers in this field to investigate the possibilities of using a plastic film as a dressing material (Hogeman & Olow, 1953; Hogeman & Olow, 1953; Knudsen, 1953; Rob & Eastcott, 1954; Lindgren, 1954; Sabbatani, 1955).

In our trials we used a specially modified acrylic resin in ethyl acetate (Nobecutan, a plastic dressing supplied by AB Bofors, Nobelkrut). Evaporation of the solvent was found to leave behind a film that appeared to fulfil the requirements of an acceptable dressing. It was therefore decided to investigate the physical and microbiologic properties of the film.

The plastic materials studied consisted of polymethacrylic acid esters and they were built up essentially according to the following formula:



[1] The preparation is described in Norwegian Patent No. 84442.

After trying a number of modifications, we found two types suitable for the purpose under consideration. One (R) of them contained an alkyl group (n-butyl), the other, an alkoxy group (2 ethoxy-ethyl). These two types are referred to below as R_1 and R_2.

PHYSICAL STUDIES

Pre-knowledge of the mechanical properties of the film is of great importance in the evaluation of the material as a wound dressing. Therefore, films obtained on evaporation of the solvent were studied for their tensile strength, ductility, fatigability (after repeated folding) and permeability to water and saline.

The films were always produced by allowing the plastic solution to evaporate in the air and then drying the film thus obtained in a thermostat for 4 days at + 35° C, and then at room temperature for at least 1 day.

Tensile strength and elasticity

The films were tested in a Schopper tensiometer with a traction rate of 0.1—1.5 mm/sec. The film specimens actually subjected to stress measured 10 mm by 10 mm. Three different thicknesses were tested (Table I).

The pull necessary to rupture the film and the elongation at rupture were noted. Both films (R_1 and R_2) were found to be more or less equally ductile, but R_1 proved

Table I. *Tensile strength and ductility of the plastic film.*

Product	Thickness of film mm			Tensile strength				Ductility		
	Mean value 20 tests	Highest value	Mean value	Lowest load, 20 tests kg	Highest load kg	Lowest load kg	Load kg/cm² original section area	Length at breakage mean value 20 tests mm	Highest value mm	Lowest value mm
R^1	0.116	0.125	0.110	1.28	1.6	0.9	110	90	105	79
R^1	0.161	0.170	0.152	1.63	1.9	1.1	101	97	108	73
R^1	0.189	0.210	0.170	1.60	2.0	1.2	84	104	125	83
R^2	0.127	0.139	0.118	0.26	0.4	0.1	20	66	105	40
R^2	0.139	0.150	0.125	0.31	0.4	0.2	22	—*)	>130	72
R^2	0.148	0.160	0.140	0.34	0.4	0.3	23	—*)	>130	95

*) In these cases the apparatus used did not permit reading of the highest values.

about 5 times stronger than R_2. On the other hand the elasticity of R_2 proved more dependent on the thickness of the film than did that of R_1. Both types of film could be stretched 6—10 times their original length before breaking. They were thus more than plastic enough for use as a wound dressing. The tensile strength of such films is probably of less importance because in reality they will hardly ever be subjected to a pull of the size used in the tests.

Fatigue on folding

The tests were carried out in a double folding apparatus of the type used in the paper industry (145 folds per minute). The film specimens had a thickness of about 0.15 mm. Owing to the plasticity of the film it was not fatigued by folding but by bending.

Type R_2 proved far superior to R_1, in that it tolerated 5000 bends (90° in either direction) without breaking, the corresponding figure for R_1 being 1200. The rate of fatigue of both types must be regarded as filling the most stringent requirements of a satisfactory wound dressing.

Permeability to water and saline

a. *Water.* A 60 mm metal tube with a bore of 26 mm was sealed at one end with the film to be tested. The tube was then filled with 2.5 g water. The other end was carefully sealed with a rubber stopper. In order to prevent any bulging of the film on insertion of the stopper, the latter was provided with a capillary tube that was sealed by fusion before the commencement of the test. The metal tubes with the plastic film attached to the downward end were suspended vertically in a drying owen at 35° C. The amount of water evaporated was afterwards determined by weighing the tubes. As far as diffusion of water is concerned, R_2 proved superior to R_1 (Table II). It also appears that the rate of diffusion varied considerably

Table II. *Permeability to water of the plastic film.*

Test No.	Type	mg H_2O/ 72 hours	Thickness of film mm	mg H_2O/ 72 hours, converted to film thickness acc. col. 6	Film thickness for which col. 5 has been corrected mm	Area of film cm^2	Evaporation per cm^2/ 24 hours in mg	Mean value mg/cm^2/ 24 hours
2	R^1	246.2	0.106	261.0	0.100	5.3	16.4)	17.3
3	»	281.6	0.096	270.3	0.100	»	15.9)	
4	»	270.3	0.115	310.1	0.100	»	19.5)	
1	»	189.6	0.139	175.7	0.150	»	11.1)	
2	»	149.1	0.150	149.1	0.150	»	9.4)	10.1
3	»	173.0	0.139	160.3	0.150	»	10.1)	
4	»	165.6	0.141	155.7	0.150	»	9.8)	
1	»	122.0	0.197	120.1	0.200	»	7.6)	
2	»	126.2	0.204	128.7	0.200	»	8.1)	7.9
3	»	121.8	0.209	127.3	0.200	»	8.0)	
4	»	111.3	0.229	127.4	0.200	»	8.0)	
1	R^2	1080.0	0.121	1306.8	0.100	7.0	62.2)	
2	»	1813.6	0.077	1396.5	0.100	»	66.5)	61.9
3	»	1691.6	0.069	1167.2	0.100	»	55.6)	
4	»	1658.3	0.080	1326.0	0.100	»	63.2)	
1	»	735.7	0.158	774.9	0.150	»	36.9)	
2	»	854.8	0.154	877.6	0.150	»	41.8)	
3	»	890.7	0.139	758.7	0.150	»	36.1)	39.3
4	»	840.4	0.144	806.8	0.150	»	38.4)	
5	»	798.2	0.170	904.6	0.150	»	43.1)	
1	»	638.1	0.206	657.4	0.200	»	31.3)	
2	»	553.8	0.223	617.4	0.200	»	29.4)	31.6
3	»	798.4	0.180	718.6	0.200	»	34.2)	

with the thickness of the film. Since a dressing film should preferably be about 1 mm thick, only the values found for this thickness are discussed below. The average rate of diffusion of water through a 1 mm film was found to be 17.3 mg/cm^2 per day, which corresponds to 294 g/day for a body surface of 1.7 m^2.

The corresponding values for R_2 were: 61.9 mg/cm^2 and 1052 g/day.

b. *Permeability to isotonic saline.* A glass tube (d = 420 mm) was sealed at one end with a plastic film about 0.15 mm thick 200 g of physiologic saline (0.90 per cent NaCl) was poured into the tube at the same time as it was immersed in running water. The tube was submerged just deep enough for the surface of the saline to be on the same level as the running water. Analysis of a sample taken 4 hours later showed a salt content of 0.91 per cent and 24 hours later 0.90 per cent. The experiment thus showed that, practically speaking, no salt diffused through the film to running water.

Adhesiveness

The power of adhesion of the film to skin was assessed by fastening a round steel plate (diameter 20 mm) with rounded edges to the skin. The plate was first pressed hard aginst the skin in order to prevent any plastic solution from penetrating between the plate and the skin. Plastic solution was then applied to the plate and the surrounding skin with a brush. All of the tests were carried out with the same size of plate, the same amount of plastic solution, the same skin area and at practically the same site on the forearm. At a varying interval after application of the solution a pull was applied at right angles to the plate which was provided with an eye for this purpose. In these tests the film regularly broke around the circumference, but never loosened from the skin. As was the case with the tensile strength, the resistance of the film of R_1 type proved greater than that of R_2, though the difference here was not so great.

The tests thus showed that the adhesive qualities of the film were satisfactory. Since the values recorded were only approximate, they are not included in the tables.

BACTERIOLOGIC STUDIES

For such a film to be acceptable as a dressing material, the solution — and the subsequent film — must be sterile. Repeated tests during a period of 3 years failed to demonstrate the presence of micro-organisms in the plastic solution.

We nevertheless considered it wise to incorporate an antiseptic in order to guarantee the sterility of the solution and of the subsequent film, and to get films with antiseptic properties.

In these investigations we tried various chemotherapeutic, antibiotic and disinfectant agents. The effect of these agents on gram positive and gram negative bacteria and fungi was also studied. Foter & Nisonger (1950), Dubos (1952).

The following micro-organisms have been tested: *Aspergillus niger (Asp. nig.), Bacillus subtilis (Bac. sub.), Candida albicans (Can. alb.), Escherichia coli (Esc. col.), Micrococcus pyog. v. albus (Mic. alb.), Micrococcus pyog. v. aureus (Mic. aur.), Penicillium roqueforti (Pen. roq.), Proteus vulgaris (Pro. vul.), Pseudomonas aeruginosa (Pse. aer.), Salmonella enteritidis (Sal. ent.), Salmonella pullorum (Sal. pul.), Sarcina flava (Sar. fla.), Sarcina lutea (Sar. lut.), Serratia marcescens (Ser. mar.), Streptococcus faecalis (Str. fae.), Streptococcus pyogenes (Str. pyo.), Torulopsis utilis (Tor. uti.), and Trichopyton interdigitale (Tri. int.).*

Only BBL preparations were used as culture media. Baltimore Biological Laboratory (1950).

As a rule, the bacteria were cultivated in broth. After incubation the number of bacteria was determined in a hemocytometer. The suspension was diluted to desired concentration with sterile, distilled water (50 000—500 000/ml).

The fungi were cultivated on slants. After spore formation suspensions were prepared as described above. Cell suspensions were prepared from yeast fungi.

The following test methods were used.

a. Agar cup method. McCulloch (1946), The Pharmaceutical Soc. (1952).
b. Agar drop plate method.
c. Agar film plate method.
d. *In vivo* disinfection tests. Stormy (1952).
e. Broth dilution method.
f. Sterility test. Price (1950), U. S. Pharmacopea (1950).
g. Other test methods (see below). NNR (1954).

ANTI-MICROBAL PROPERTIES OF THE PLASTIC SOLUTION WITH AND WITHOUT ADDITION OF ANTISEPTICS

Without addition of antiseptic agents

The plastic solution was studied for any antimicrobial properties by the agar drop

Table III. *"Screening-tests" with plastic solutions different anti-microbial substances (inhibition zones).*

«Agar drop-plate method»	per 0.1 ml solution	Bac. sub.	Esc. col.	Mic. aur.	Pro. vul.	Sal. ent.	Sal. pul.	Sar. fla.	Str. fae.
Bacitracin	25 µg	0	(0)	(0)		(0)			(0)
»	125 »	0	(0)	4			0	19	
Chloromycetin	50 »	(0)	2	0		6			6
»	125 »	7	10	12			(0)	18	
Benzyl-Penicillinnatrium	50 »	(0)	(0)	20		(0)			5
» »	45 »	0	(0)	16			0	(0)	
Procain-Penicillinnatrium	45 »	0	(0)	0			0	(0)	
Terramycin . HCl	125 »	0	9	11			5	11	
Aureomycin . HCl	125 »	9	11	18			7	15	
Bacitracin + Chloromycetin	25 » + 50 »	0	1	0		3			5
Bacitracin + Neomycin + Nebocetin	125 » + 75 »	6	3	5			12	19	
Penicillin + Streptomycin	50 » + 50 »	(1)	(1)	23		(2)			(3)
Penicillin + Streptomycin + Streptopenin	45 » + 125 »	10	12	8			0	9	
Dihydrostreptomycinsulphate	50 »	0	(0)	1		2			(1)
—»—	125 »	6	19	1			0	10	
Penicillin + Terramycin	25 » + 20 »	0	13	36	3	9	12	16	12
Sulphatiazol	5000 »	(22)	(0)	(4)		(0)			(0)
Nitrofurazone	500 »		4	7	0	12			2
Desivon (Benzalkoniumchloride)	100 »	0	(0)	6			(0)	10	
»	250 »	0	0	7			6	11	
Tetramethylthiuramdisulphide	50 »		4	15	1	1			5
»	100 »		10	18	5	8			10
»	250 »	(21)	16	22			20	23	
»	250 »	18	13	22	10	10	31		26
»	500 »		21	27	16	19			24
»	500 »		22	27	17	19			20

plate method. Test micro-organisms: *Bac. sub., Esc., col., Mic. aur., Pro. vul., Pse. aer., Sal. pul., Sar. fla., Ser. mar., Str. fae.,* and *Tor. uti.*

The investigation showed that the plastic solution has no anti-microbial properties, micro-organisms regularly growing even beneath the film.

Then the solvent and the film were studied separately by the agar cup method and the agar film plate method, respectively. Test organisms: *Bac. sub., Esc. col., Mic. aur., Sal. ent., Ser. mar., Asp. nig., Pen roq.,* and *Tor. uti.* This investigation showed that the solvent (ethyl acetate) may be regarded as slightly bacteriostatic, while the acrylic film possesses no anti-bacterial properties. Therefore, the incorporation of a suitable antiseptic was considered desirable in order to keep the skin aseptic.

A study of the effect against anaerobic bacteria is in progress.

Table IV. *Effects of various microbicides in plastic films in the presence of serum.*

«Agar film-plate method» 10 × 10 × 0.1 mm		Bac. sub.	Esc. col.	Mic. aur.	Sal. pul.	Sar. fla.
Penicillin	N—A	(0)	(0)	(0)	(0)	(0)
15 μg	Se—A	(0)	(0)	(0)	(0)	(0)
Streptomycin	N—A	9	11	13	(0)	17
42 μg	Se—A	19	17	14	5	22
Streptopenin	N—A	16	5	15	(0)	16
57 μg	Se—A	(0)	(0)	13	(0)	13
Bacitracin	N—A	(5)	(0)	13	5	21
42 μg	Se—A	(0)	(0)	7	(0)	21
Nebocetin	N—A	7	5	5	6	19
67 μg	Se—A	10	5	(0)	(0)	13
Aureomycin	N—A	18	26	12	20	25
42 μg	Se—A	(0)	(0)	9	(0)	14
Chloromycetin	N—A	5	7	5	(0)	15
42 μg	Se—A	5	5	(5)	5	15
Terramycin	N—A	5	(0)	(0)	(0)	14
42 μg	Se—A	(0)	(0)	(0)	(0)	13
TMTD	N—A	11	14	20	22	26
83 μg	Se—A	19	5	13	15	16

Table V. *Microbicidal properties of plastic solution containing 0.25 per cent TMTD.*

Micro-organism	Asp.nig.	Bac.sub.	Can.alb.	Esc.col.	Mic.alb.	Mic.aur.	Pro.vul.	Pse.aer.
Inhibition zone	35	21	17	13	21	30	20	8
Micro-organism	Sal.ent.	Sal.pul.	Sar.fla.	Sar.lut.	Ser.mar.	Str.fae.	Str.pyo.	Tri.int.
Inhibition zone	10	24	23	27	10	14	26	21

Screening tests with plastic solutions containing various anti-microbial agents

A large number of plastic solutions were prepared with the addition of chemotherapeutics, antibiotics, quaternary ammonium compounds and other disinfectant agents. The active components were tried in various concentrations. Sometimes combinations of such agents were also tried. The solutions were tested on various types of micro-organisms in order to study their effect and to assess the optimal concentration. The results of these screening tests on ordinary agar plates are accounted for in Table III.

The figures in Table III denote the difference between the diameter of the bacteria-free zone and that of the drop.

Table VI. *Microbicidal effect of plastic solution without and with the addition of TMTD. (Growth: +. No growth: —)*

Contact period	1 min.		30 mins.		2 hours		7 hours		24 hours	
Incubation period (hours)	24	48	24	48	24	48	24	48	24	48
Artificial contamination with 10 mio *Bac.sub.* per 10 ml plastic solution and water respectively										
Plastic solution only	—		—	++	—	++	—	++	—	++
Plastic solution with 0.25% TMTD	—	—	—	—	—	—	—	—	—	—
Water	++		++		++		++		++	
Artificial contamination with 10 mio *Esc.col.* per 10 ml plastic solution and water respectively										
Plastic solution only	—	—	—	—	—	—	—	—	—	—
Plastic solution with 0.25% TMTD	—	—	—	—	—	—	—	—	—	—
Water			++		++		++		++	
Artifical contamination with 10 mio *Mic.aur.* per 10 ml plastic solution and water respectively										
Plastic solution only	—	+	—	—	—	—	—	—	—	—
Plastic solution with 0.25% TMTD	—	-	—	—	—	—	—	—	—	—
Water	++		++		++		++		++	

Contact period	1 min.		30 mins.		2 hours		7 hours		24 hours	
Incubation period (hours)	48	72	48	72	48	72	48	72	48	72
Artificial contamination with 10 mio Asp.nig.-spores per 10 ml plastic solution and water respectively										
Plastic solution only	—	++	—	—	—	—	—	—	—	—
Plastic solution with 0.25% TMTD	—	—	—	—	—	—	—	—	—	—
Water			++		++		++		++	

All values are the means of 2—10 tests. (o) indicates bacterial growth beneath the film, the figures in the brackets indicating an uncertain zone of inhibition. It is apparent from the table that the films containing these agents had a varying effect on the different micro-organisms.

In order to study the effect of serum, films containing various anti-bacterial substances were prepared and tested by the agar film plate method. The films were 10 mm by 10 mm and 0.1 mm thick. The test organisms and the results are apparent from Table IV.

(For explanation of values, see Table III.)

N-A denotes nutrient agar BBL and Se-A, serum agar BBL.

As is apparent, TMTD (Tetramethylthiuram disulphide) had the strongest effect and on the widest range of micro-organisms. Henn (in press).

Table VII. *Depot effects of films containing TMTD.*

«Agar film-plate method» *Mic. aur.*	Effective inhibition zones	
At the start	15	14
After 24 hours → new Agar plate	15	14
After a further 24 hours → new Agar plate	10	10
After a further 24 hours → new Agar plate	11	11
After a further 24 hours → new Agar plate	6	5
After a further 24 hours → new Agar plate	3	4

Table VIII. *Washing tests with films containing TMTD.*

Washing time (hours)	0	1	3	6	24	48
«Washing material»	Effective inhibition zones as compare to *Mic.aur.*					
Running water, 30°C	18		13	11	8	
Running water, 37°C	24	23	17	16	9	11
Physiological solution of common salt, 37°C	24	22	17	16	12	10

Table IX. *Stability investigations of plastic solution containing TMTD.*

Test organisms	Esc. col.	Mic. aur.	Pse. aer.	Sal. ent.	Asp. nig.
Storage conditions	Inhibition zones according to Agar drop-plate method				
Newly prepared solution	15	36	12	11	34
5 months, dark, at 22°C	14	31			32
6 months, sunlight, at 25°C	0	23	0	0	16
13 months, dark, at 6°C	14	35	11	10	30

TMTD as an antiseptic for plastic solution

In view of the results obtained we considered further to study the effect of the incorporation of the TMTD on the plastic film desirable. We first extended the screening tests for the effect of TMTD only. The results are given in Table V.

It is apparent that the plastic film containing TMTD had a strong effect on all of the bacteria and fungi tested.

TMTD compared with skin antiseptics

In order to estimate the disinfecting power of TMTD we carried out trials with Stormy's *in vivo* test. Stormy (1952). For comparison, 70 per cent alcohol and 0.1 per cent tincture of iodine was used. Test organisms: *Mic. aur.* and *Esc. col.*

0.25 per cent TMTD in ethyl acetate-polyglycol (3:2) produced the same effect as 70 per cent alcohol and 1 per cent tincture of iodine, but a better effect than 0.10 per cent desivon.

The dermatologic and toxicologic properties of TMTD will be the subject of a separate paper (Henn, in press).

Effect of TMTD as judged by the broth dilution method

No growth resulted with 5 ppm. TMTD in nutrient broth against *Mic. aur.* and 100 ppm. TMTD against *Esc. col.* Moderate growth with 1 ppm. and heavy growth with 0.1 ppm. was the result against *Mic. aur.* The corresponding values against *Esc. col.* were 50 ppm. and 5 ppm., respectively. The addition of 10 per cent horse serum did not influence the results. (Effect of antibiotics, as judged by the broth dilution method, see The Pharmaceutical Soc. (1952).)

Sterility test of plastic solution with and without addition of TMTD

The plastic solution was tested by the method described in USP XIV (1950).

As pointed out on page 278 in no instance was bacterial growth demonstrable.

Then the plastic solution was contaminated intentionally by the addition of suspensions of micro-organisms (10 mill. bacteria or spores per 10 ml) and samples were obtained after varying contact periods for cultivation on broth. The test organisms used and the results obtained are given in Table VI.

Further tests with plastic film containing TMTD

In order to study how long a plastic film with TMTD retains its bactericidal properties, films were prepared and tested by the agar film plate method. The zone of inhibition was measured after 24 hours, after which the film was transferred to a new plate, *etc.* The results are given in Table VII.

The figures denote the zone of effective inhibition.

The experiments show that the film retains its bactericidal effect for 4—5 days before any appreciable reduction is demonstrable. We interpreted the results as a sign that TMTD diffuses out through the plastic film. If this interpretation is correct, the question arises whether it might not be possible to accelerate the diffusion by some means or other. Can *e. g.* rinsing of the film with water or saline be of any importance in this respect? In order to test this possibility, films were allowed to hang under running water or saline for a varying period and than applied to inoculated agar plates. As is apparent from Table VIII, (test organisms: *Mic. aur.*) the TMTD could only slowly be washed out of the film.

STABILITY OF THE BACTERICIDAL EFFECT OF THE PLASTIC SOLUTION CONTAINING TMTD

Does the bactericidal effect of the solution with TMTD deteriorate with storage of the solution? Are the storage qualities influenced by external factors?

In an attempt to find answers to these questions, plastic solutions were prepared and stored for a varying period under relatively different circumstances. They were then subjected to routine tests by the drop plate method. The results are given in Table IX.

For explanation of figure, see Table III.

The solutions were stored in ordinary transparent bottles. It is apparent from the table that they could be stored practically indefinitely as long as they were kept in the dark. When exposed to the light, however, the effect of the TMTD soon disappeared, probably because of photochemical decomposition. The solution with TMTD should therefore be stored in the dark.

SUMMARY

1. The physical properties of two different plastics (R_1 and R_2) of polymethacrylic type were studied for their value as a dressing material. The polymers used were prepared with special regard to the object of obtaining a highly elastic film readily permeable to water vapour. The permeability of the polymethacrylic film was increased by the introduction of an ether bridge in the

alkyl part of the alcohol group (R_2). This type of polymer (R_2) was also very elastic.

2. These plastic materials dissolved in ethyl acetate are sterile and sligthly bacteriostatic. The plastic films are sterile but possess no anti-microbic properties.

3. Plastic solutions and films containing TMTD are sterile and strongly microbicidal.

TMTD as a disinfectant is soluble and stable in plastic material of the polymethacrylic ester type and gives the plastic film good microbicidal properties against gram positive and gram negative bacteria and against fungi. TMTD is also active under anaerobic conditions and in the presence of proteinic substances.

5. TMTD as an addition to plastic films was found to possess better microbicidal properties than a large number of other antibotics and disinfectants of conventional type.

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DIAGNOSIS OF PHEOCHROMOCYTOMA BY FLUORIMETRIC ESTIMATION OF ADRENALINE AND NORADRENALINE IN URINE

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In 1950 Engel & Euler described two cases of hypertension, both secreting large amounts of noradrenaline in urine, one of them adrenaline also. In both cases a pheochromocytoma was found and removed. The diagnostic value of this finding was pointed out and the method of urine analysis has since been applied by several authors (Lund, 1952; Burn, 1953; Goldenberg, Serlin, Edwards & Rapport, 1954; Pekkarinen & Pitkänen, 1955 a, b). While other, indirect methods not infrequently give false positive or false negative results (mechanical provocation, drug tests with histamine, benzodioxane, mecholyl, dibenamine and phentolamine *etc.*) the urine catechol amine analysis has the advantage of giving direct information about the pathogenic factor. Moreover this method does not incur any risk or inconvenience to the patient. An evaluation of the present diagnostic and therapeutic measures in pheochromocytoma will be presented elsewhere (Euler & Ström, 1956). In view of the practical importance of the urine analysis for ascertaining the diagnosis of pheochromocytoma, a relatively simple method which permits clinical routine laboratory estimation of noradrenaline and adrenaline in urine has been elaborated and is described in the present report.

PREVIOUS METHODS

Several methods for the assay of catechol amines in urine which may be used for the diagnosis of pheochromocytoma have been reported previously (Engel & Euler, 1950; Euler, 1951; Lund, 1952; Goldenberg, Serlin, Edwards & Rapport, 1954; Pekkarinen & Pitkänen, 1955; Euler & Floding, 1955; Moulton & Willoughby, 1955 and others). Although all of these methods may serve the purpose, they are in some respects unsatisfactory as regards accuracy, specificity or practical usefulness. Not even the simple screening method using untreated urine on biological test preparations can be done in all laboratories, and most of the methods based on chemical estimations are relatively complicated or do not allow differentiation between adrenaline and noradrenaline.

Colorimetric methods cannot be applied to urine extracts containing normal amounts of catechol amines, and only give satisfactory results with urines from some cases of pheochromocytoma.

The present method is chiefly based on the technique described previously by Euler & Floding (1955), using adsorption of catechol amines on aluminum oxide, and fluorimetric estimation in the eluate of the trihydroxyindole compounds obtained by oxidation with ferricyanide, transformation with alkali and stabilization with ascorbic acid according to Ehrlén (1948).

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TECHNIQUE

Collection of urine

Urine is collected preferably for 24 hours, acidified with hydrochloric or sulfuric acid to pH 2—3 and kept cold. It has been found that urine so treated retains its catechol amines for a long time. Even at room temperature no loss of catechol amines was observed in a week when the urine was acidified. Samples with a pH of 6 and above often show growth of bacteria on standing which may turn the reaction alkaline, causing inactivation of the catechol amines.

Of the collected 24 hour urine approximately 1/10 (50—100—200 ml) is adjusted to about pH 3, (estimated with pH-paper), briefly heated to boiling and filtered.

For the adsorption of the catechol amines, acid-washed aluminum oxide is used. Preparations from Merck & Co. and from British Drug Houses have been used. 200 g Al_2O_3 are boiled in 1 litre 2 N HCl for 20 minutes and filtered on a suction funnel, thoroughly washed with distilled water until all acid is removed, and dried. It is kept in a tightly stoppered bottle.

To the urine sample, usually 100 ml, is added 2.5 g aluminum oxide and 1 g ethylene diaminetetraacetic acid (EDTA). It has been found essential for the differentiated estimation of adrenaline and noradrenaline to add this substance. It has no apparent influence on the result of chemical estimation of the total catechols or in the biological tests, but its presence prevents the often occurring oxidation of noradrenaline at pH 3.5, which gives too high apparent adrenaline figures.

For the adsorption of the urine catechols on Al_2O_3, 0.5 N NaOH is added dropwise with vigorous stirring, for instance by the aid of a magnetic stirrer. By adding 0.1 ml 1 per cent brom thymol blue in ethanol to the urine the neutral point is easily recognized and the titration is then carried on to pH 8.5. At this point which is checked with Merck Special indicator paper pH 8.2—10.0 or Lyphan paper 8.2—9.7 the adsorption of the catechol amines is completed.

The suspension is then filtered on a suction funnel, 5 cm in diameter, on papers allowing a rapid flow of clear solution. The residue is washed twice with 10 ml distilled water. Insufficient washing may result in an alkaline first eluate with consequent inactivation of the catechol amines. The Al_2O_3 is then eluted with 2×4.5 ml 0.25 N sulfuric acid, and the eluates collected in a second small suction flask, to which 0.05 ml 0.04 per cent brom phenol blue has been added. A 10 per cent solution of sodium bicarbonate is then added until faint blue color (pH about 4). The eluate is then made up to 10 ml with distilled water. The solution may be stored in this state in a refrigerator for at least a week or in a deep freeze for months without deterioration.

FLUORIMETRIC ESTIMATION OF ADRENALINE AND NORADRENALINE

The fluorimetric estimation can be made conveniently with a Coleman Photofluorometer model 12 C, which is sufficiently sensitive and stable for the purpose.

Principle

The procedure is based on the formation of trihydroxyindoles on oxidation with ferricyanide, rearrangement with strong alkali and stabilization of the fluorescent compound with ascorbic acid (Ehrlén, 1948). The fluorescent product is stable for a period of up to an hour. Adrenaline and noradrenaline are oxidized differentially at different reactions. At pH 6 both amines are oxidized, at pH 3.5 adrenaline and a small part, about 4 per cent of the noradrenaline (Euler & Floding, 1955).

Procedure

Reagents:

Buffer solutions: 1 M acetic acid-sodium acetate pH 3.5 and 6.0.

Potassium ferricyanide	0.25	per cent
Zinc sulfate	0.5	» »
Sodium hydroxide	20	» »
Ascorbic acid	2	» »

Combined reagent: 4.5 ml of the NaOH solution is mixed immediately before use with 0.5 ml ascorbic acid solution, prepared the same day.

1. *Estimation of adrenaline plus noradrenaline.*

The reaction is carried out in a graduated 10 ml cylinder as follows. To 0.1—0.5 ml of the clear eluate, (filtered or centrifuged if necessary) is added 1 ml 1-molar acetate-buffer pH 6.0 and 0.1 ml 0.25 per cent potassium ferricyanide solution which is allowed to act for 2 minutes. After this time 1 ml of the sodium hydroxide-ascorbic acid mixture is added and distilled water to 10 ml, and the fluorescence determined as described below.

2. *Adrenaline.*

The reaction is carried out as above with the following differences. To the same amount of the extract is added 1 ml acetate buffer at pH 3.5, 0.1 ml 0.25 per cent potassium ferricyanide and 0.4 ml 0.5 per cent zinc sulfate. After 3 minutes 1 ml of the sodium hydroxide ascorbic acid mixture is added and the volume made up to 10 ml.

3. *Reagent blank.*

The reagent blank includes all reaction solutions.

4. *Extract blank.*

All reagents are added to the sample with the exception of the ferricyanide. Any adrenochrome or noradrenochrome present in the sample will then be rearranged and stabilized as fluorescent trihydroxyindole compound.

5. *Standards.*

For standards 0.2 μg noradrenaline HCl or 0.1 μg adrenaline HCl are used and the reactions carried out as above, except that 0.1 ml zinc sulfate was used at pH 3.5.

Fluorimetric estimation

The Coleman Photofluorometer was used with primary filter Schott BG12, blue-green, max. transmission 435.8 mμ and secondary filter GG 11, yellow-green, transmission limit about 500 mμ and the fluorescence of the solutions measured against a standard. The sensitivity is adjusted to give a deflection of 50 scale divisions, plus the deflection caused by the reagent blank, for 0.2 μg noradrenaline. As standard a solution of 10—20 μg/100 ml quinine sulphate may be used which is compared with an adrenaline or nordadrenaline standard. More convenient is to use a perspex rod as standard, inserted in a fluorimeter test tube.

A linear function is obtained for increasing concentrations on the scale.

The deflections are recorded 1) for the extract blank 2) for noradrenaline + adrenaline (pH 6) and 3) for adrenaline + a small amount of noradrenaline (pH 3.5).

Calculation of results

The following symbols are used:

Amount of noradrenaline standard corresponding to 100 scale divisions ..... = a

Percentage noradrenaline oxidized at pH 3.5 in 3 min. ..... = n

(The fluorescence intensity of noradrenaline oxidized at pH 3.5 is approximately 4 per cent of that obtained at pH 6).

Reading for adrenaline + noradrenaline pH 6) = A

Reading for adrenaline + 4 per cent noradrenaline (pH 3.5) ..... = B

Extract blank (pH 6.0) ..... = E

Relative fluorescence intensity for equal amounts of adrenaline and noradrenaline = m

(The relative fluorescence intensity for equal amounts of noradrenaline and andrenaline is 1:1.6 using the above filters).

Hence:

$$\text{Noradrenaline} = y = \frac{a(A-B)}{(100-n)}$$

$$\text{Adrenaline} = x = \frac{a(B-E)}{100\ m} = \frac{n \cdot y}{100\ m}$$

Using a value of 1.6 for m, a standard amount of noradrenaline of 0.4 μg (a), and 4 per cent oxidation of noradrenaline at pH 3.5 (n), the expressions will read, in μg:

$$\text{Noradrenaline} = y = 0.42 \cdot \frac{(A-B)}{100}$$

$$\text{Adrenaline} = x = 0.25 \cdot \frac{(B-E)}{100} - 0.025\,y$$

It has also been possible to use Beckman spectrophotometer model DU when fitted with a high pressure mercury vapour lamp. With standard cuvettes this arrangement gives satisfactory readings for most samples. When fitted with a photomultiplicator the sensitivity is adequate even for weak fluorescence intensities.

COMPARISON OF RESULTS OF BIOLOGIAL ASSAY AND FLUORIMETRIC ESTIMATION

The adrenaline and noradrenaline content of 50 urine samples from various clinical cases including hypertension and 10 cases

Table Ia. *Urinary excretion of adrenaline in mμg/min. estimated biologically and fluorimetrically in 50 unselected cases.*

No.	Biol.	Fluor.	Diff.	No.	Biol.	Fluor.	Diff.
13	0	0	0	55	2.2	5.6	3.4
26	0	1.4	1.4	30	2.7	2.4	—0.3
28	0	0.52	0.52	39	2.8	3.2	0.4
31	0	1.7	1.7	49	2.8	4.0	1.2
38	0	1.5	1.5	42	2.8	7.4	4.6
25	0	1.6	1.6	8	2.9	6.2	3.3
41	0	0	0				
			average 0.96				average 2.1
			(0—1.7)				(—0.3—4.6)
23	0.27	1.8	1.5	14	3.0	3.3	0.3
22	0.30$_1$	1.9	1.6	12	3.1	5.6	2.5
2	0.43	3.4	3.0	9	3.2	4.5	1.3
52	0.61	3.5	2.9	37	3.3	4.1	0.8
1	0.64	2.9	2.3	7	3.5	3.3	—0.2
6	0.76	1.9	1.1	46	3.5	4.2	0.7
56	0.83	1.9	1.1	43	3.9	4.4	0.5
			average 1.9				average 0.84
			(1.1—3)				(—0.2—2.5)
24	1.1	4.8	3.7	27	4.0	9.0	5.0
32	1.3	4.5	3.2	33	4.9	5.6	0.7
34	1.3	0.9	—0.4	35	5.0	6.7	1.7
48	1.4	2.2	0.8	21	5.2	10	4.8
50	1.5	2.6	1.1	29	5.2	4.8	—0.4
5	1.7	4.4	2.7	44	5.3	9.0	3.7
40	1.7	4.0	2.3	4	5.3	6.5	1.2
47	1.8	3.1	1.3	36	5.6	6.2	0.6
10	1.9	1.9	0				
19	1.9	2.8	0.9				average 2.2
			average 1.6				(—0.4—5.0)
			(—0.4—3.7)	20	6.2	9.2	3.0
				15	6.9	10	3.1
				45	7.9	14	6.1
				51	9.0	11	2.0
				11	12	13	1.0
							average 3.0
							(1.0—6.1)

Table I b. *Urinary excretion of noradrenaline in mμg/min. estimated biologically and fluorimetrically in 50 unselected clinical cases.*

No.	Biol.	Fluor.	Diff.	No.	Biol.	Fluor.	Diff.
39	1.1	3.5	2.4	33	15	36	21
34	3.3	5.1	1.8	47	15	16	1
			average 2.1	30	16	21	5
			(1.8—2.4)	28	17	23	6
48	5.6	18	12	51	17	24	7
52	6.0	16	10	56	27	27	8
40	6.2	13	6.8	1	19	32	13
4	6.9	16	9.1	2	19	27	8
19	6.9	11	4.1	43	19	28	9
9	7.6	13	5.4	44	19	21	2
10	7.6	12	4.4				average 7.8
46	7.6	17	9.4				(1-21)
6	9.0	9.7	0.7	36	22	17	—5
7	9.7	13	3.3	20	23	37	14
			average 6.5	21	23	28	5
			(0.7—12)	25	25	33	8
8	10	30	20	27	26	32	6
22	11	15	4	41	29	28	—1
5	11	15	4				average 4.5
11	12	24	12				(—5-14)
12	12	19	7	32	35	40	5
50	12	18	6	31	43	40	—3
23	13	14	1	45	43	41	—2
35	13	19	6	13	46	44	—2
37	13	22	9	24	53	77	24
49	13	17	4	42	61	72	11
55	13	25	12	14	64	84	20
26	14	22	8	38	69	67	—2
29	15	22	7				average 6.4
							(—3-24)
				15	123	159	36

of verified pheochromocytoma has been determined by biological assay (cat's blood pressure and chicken rectal caecum) (Euler, 1949) and with the present method using the Coleman fluorimeter. All figures are given in terms of the hydrochlorides.

The results are given in Tables I a, I b and II in which the material has been arranged in groups with increasing figures obtained with the biological method and compared with the fluorimetrically found figures.

For each group the average difference and range has been given.

In Table II the percentage difference between the two methods is also given for the noradrenaline figures.

DISCUSSION

A comparison between the adrenaline and noradrenaline figures obtained with the two methods shows a good general agreement as to the order of magnitude of the amounts estimated.

The reason for the discrepancies between the figures obtained by fluorimetric estimation and by biological assay cannot be stated with certainty. It is conceivable, however, that certain errors are inherent in both methods, apart from the error of estimation, which is considerable for the biological method but small for the fluorimetric method. In the biological assay method it can not be excluded that biologically active catechol amines other than adrenaline and noradrenaline are present and may affect the results. Dopamine is known to occur in the urine and will be present in the adsorbed extracts (Holtz, Credner & Kroneberg, 1947; Euler, Hamberg & Hellner, 1951; Euler, Euler & Floding, 1955, and others).

Although the biological activity of dopamine is only 1/50—1/100 of that of l-noradrenaline on the preparations used, the amounts may occasionally be high enough to influence the figures (Hökfelt & Goldenberg, 1956).

The amount of dopamine in normal urine, untreated or subjected to acid hydrolysis, has been estimated to 100—200 μg per 24 hours (Euler, Hamberg & Hellner, 1951). Peil-Malherbe (1956) found on an average 1 μg dopamine in acid hydrolyzed urine per litre. Using a preparation of dopamine which was kindly put at our disposal by V. Tainter, Sterling Winthrop Research Institute, its fluorescence was about 3 per cent of that of noradrenaline when the oxidation was carried out at pH 6.

Table II. *Urinary excretion of adrenaline and noradrenaline estimated biologically and fluorimetrically in 10 cases of surgically verified pheochromocytoma.*

Adrenaline mμg/min.

No.	Biol.	Fluor.	Diff.
8	0	0	0
1	1.1	1.4	0.3
2	1.5	12	11
5	2.4	20	18
6	3.0	16	13
9	5.1	13	7.9
			average 8.4 (0—18)
2a	10	13	3
3	13	19	6
			average 4.5 (3—6)
4	120	147	27
7	195	292	97
			average 62 (27—97)

Noradrenaline mμg/min.

No.	Biol.	Fluor.	Diff.	Deviation in per cent
4	113	130	17	+15
1	153	134	—19	—12
3	271	292	21	+ 8
2	288	344	56	+19
2a	428	347	—81	—19
			average — 3 (—81-56)	
5	944	770	—174	—18
7	985	1000	15	+ 1.5
9	1430	1390	—40	— 2.8
6	1460	1480	20	+ 1.3
			average —90 (—174-20)	
8	8000	10300	2300	+29

From these data it can be calculated that the error introduced by the dopamine present

in urine generally will be quite small, both in the biological and colorimetrical estimations. Since dopamine has a biological action similar in kind to that of noradrenaline but much weaker, even an amount of 200 μg per 24 hours or about 140 mμg per min. would only give an apparent noradrenaline figure of 1.4—2.8 mμg per min. biologically and 4.2 mμg per min. fluorimetrically.

A comparison of the biologically estimated and fluorimetrically found figures for adrenaline shows that the latter figures are on an average about 2 mμg higher per min.

The corresponding difference for noradrenaline is similarly relatively constant, although higher, about 6 mμg per min. In both series the difference is smaller in the groups with the lowest figures obtained by biological assay. Thus there seems to be a relatively small systematic difference between the two methods, which may have to be taken into consideration when figures obtained with the two methods are compared.

Since the increase in the urinary output of adrenaline and noradrenaline during various physiological or pathological conditions often amounts to 5—10 times the basic figure, the difference observed between the two methods can generally be regarded as negligible. This is particularly the case in pheochromocytoma where the percentage deviation for the noradrenaline figures with the two methods is relatively small. The reason for this is probably to be sought in the fact that only small amounts of the extracts need to be used, whereby unknown factors are reduced. In some cases the colorimetric method of Euler & Hamberg, (1949) has been applied to urine extracts from cases of pheochromocytoma and found to give figures in close agreement with the fluorimetric figures.

The present method seems to be accurate enough to permit a safe discrimination between normal and increased catechol amine figures in urine for the diagnosis of pheochromocytoma and at the same time sufficiently rapid and simple to find use in clinical laboratories. It may also be used for catechol amine estimations in urine in a variety of other conditions.

SUMMARY

A method for estimation of noradrenaline and adrenaline in urine, using standard fluorimetric technique, and suitable for clinical routine laboratories is described.

The excretion figures for adrenaline and noradrenaline in 10 patients with verified pheochromocytoma were from 10—100 times larger than those in a random material of 50 subjects including patients with essential hypertension.

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URINARY EXCRETION OF HISTAMINE IN HEALTHY HUMAN SUBJECTS

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It was not until recent years, notwithstanding numerous earlier attempts, that the normal occurrence of histamine in human urine was satisfactorily demonstrated (Anrep, Ayadi, Barsoum, Smith & Talaat, 1944). Both free and conjugated histamine occur, though the latter form is generally considered to preponderate. The above mentioned authors detected only traces of free histamine, whereas conjugated histamine was found in amounts between 0.09 and 5.2 μg per ml. In the opinion of Adam, Hunter and Kinnear (1951) the 24-hour urinary output of free histamine averages 8.6 μg and of conjugated histamine 29.6 μg.

Conjugated histamine may be converted by acid hydrolysis into a biologically active form, which can be quantitatively bioassayed. Urbach (1949) presented evidence that the conjugated histamine extracted from urine is acetyl-histamine. Histamine administered by mouth is excreted in conjugated form, and Urbach showed that this acetylation is probably brought about by intestinal bacteria. Histamine administered by subcutaneous injection, on the other hand, is chiefly excreted in free form (Anrep *et al.*, 1944).

Both chemical and biological methods have been employed for the determination of histamine in urine. Many authors however, regard the hitherto employed chemical methods as unreliable, because of the comparatively minute amount of histamine normally present in the urine and also because of the large number of other substances occurring there, which may influence the results. For biological assay, many workers have employed adsorption on animal charcoal at extraction, but this method has yielded extremely varying results. Insufficient attention appears to have been given to those factors which, at collection of urine samples, may affect the amount of histamine in the urine.

Adam (1950) described, in a preliminary report, a method of extracting histamine from urine by adsorption on cation exchangers, and gave the normal value for males at between 15 and 30 μg free histamine per day. Bergström & Hansson (1951) reported a method for extraction of histamine from distilled water solution with the use of an Amberlite ion exchanger. Technically the method is relatively simple and gives a high yield. The aim of the present study was to develop, on the basis of this mode of procedure, a method suitable for extraction and determination of histamine in urine.

METHODS

Collection of urine

The urine was collected in one-liter flasks containing 10 ml 2 N HCl. In this way the pH of the urine could be kept below 3.5. Great importance was attached to pouring the urine into the flasks immediately after micturition. During collection of the samples the flasks were stored at a temperature of +4° C. The day urine (0700—1900 hours) and the night urine (1900—0700 hours) were collected separately.

Extraction method

The method employed here was, with the exception of small modifications, the same as devised by Bergström & Hansson (1951) for histamine in aqueous solution.

Extraction of free histamine

Of the collected urine, 100 ml was taken and adjusted with NaOH to pH 6.5. After filtration the clear filtrate was passed through an Amberlite IRC-50 column measuring 10 by 80 mm. The ion exchanger had been pre-treated as described by Bergström & Hansson (1951). The outflow from the column was so adjusted as to give a drip rate of 2—3 ml per minute. When all urine had passed through the column the latter was washed with 2 × 25 ml distilled water. The change from urine to water was so adapted as to prevent access of air to the ion exchanger. When the water level had reached the upper edge of the ion exchanger column, the adsorbed histamine was eluted by adding 1 N HCl. The pH in the outflow was checked. When the latter showed an acid reaction, collection of the eluate was started and the elution continued with 0.01 N HCl until a total of about 10 ml acid had been supplied. The histamine was immediately liberated from the ion exchanger when the pH level shifted towards acid.

The eluate was neutralized with NaOH, and the histamine activity then assayed on isolated guinea-pig ileum. Pure NaCl solutions having the same salt concentration as that in the samples usually produced no effect on the gut, though in a few exceptions they caused slight contraction. In these latter cases the gut was discarded.

Extraction of total (free and conjugated) histamine

To 100 ml urine, with the pH adjusted to 6.5, was added 200 cc acetone. After shaking and filtration, the clear filtrate was concentrated to dryness *in vacuo* on a water bath. The residue was dissolved in 10 ml of 10 N HCl and hydrolyzed by boiling in a water bath for 1½ hours. The sample was again concentrated *in vacuo* to absolute dryness; the residue was dissolved in distilled water and, after adjustment of the pH to 6.5, was diluted with distilled water to 100 cc. The histamine was thereafter extracted by adsorption on an Amberlite column as described above in the case of free histamine.

Biological assay of histamine

The extracts were tested in isolated guinea-pig ileum in a 3 ml bath containing Tyrode's solution at 38° C. The bath fluid was aerated with pure oxygen. All samples were tested both before and after addition of an antihistaminic (Lergitin, Recip) to the Tyrode's solution. In each case the stimulating effect on the gut was totally inhibited by Lergitin. The figures for histamine are given as base values.

RESULTS

Studies of the accuracy of the method

1. Influence of the *degree of acidity* upon the adsorption of urinary histamine on Amberlite IRC-50. — An optimal yield was obtained at pH 6.5, the values progressively decreasing with rising or falling pH levels (Fig. 1).

2. Influence of the *salt concentration* upon the adsorption of histamine on the ion exchanger. — A high salt content of the solution considerably impaired the adsorption of histamine. By increasing the length of the column it was possible to obtain an increasingly higher adsorption of histamine in salt solution — The relation between salt concentration and histamine adsorption on

Table I. *Influence of salt concentration on the recovery of histamine from a water solution.*

g NaCl added to 100 ml a.d.	Histamine in μg added to 100 ml a.d.	Recovery in μg	Recovery in per cent
1	3.0	2.6	87
	5.0	4.6	93
3	3.0	2.5	84
	5.0	4.5	89
5	3.0	1.8	61
	5.0	3.4	68
10	3.0	1.3	42
	5.0	1.9	38
15	3.0	1.2	41
	5.0	1.7	35

Table II. *Recovery of histamine added to 100 ml of urine.*

Histamine added μg	Histamine found μg	Recovery in μg	Recovery in per cent
0	1.6	—	—
2.0	3.5	1.9	95
5.0	5.9	4.3	85
0	0.9	—	—
5.0	4.7	3.8	76
10.0	8.0	7.1	71
0	0.7	—	—
10.0	9.0	8.3	83
20.0	15.5	14.8	74
0	1.2	—	—
2.0	2.6	1.4	—
2.0	2.6	1.4	70
5.0	4.8	3.6	72
8.0	7.3	6.1	76
0	0.5	—	—
2.0	1.9	1.4	70
5.0	4.3	3.8	76
		Mean	77

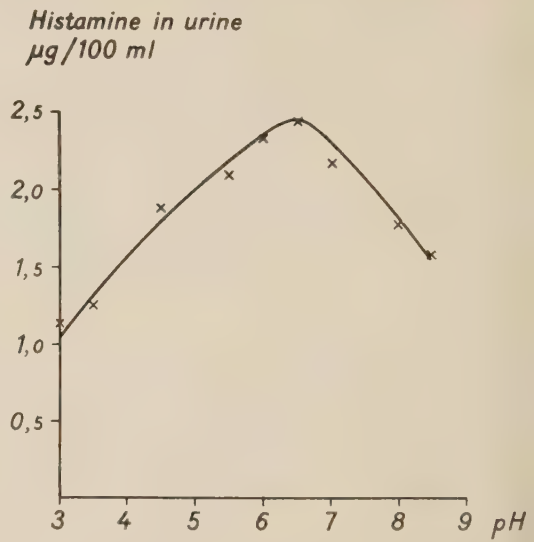

Fig. 1. The influence of pH in the urine on the histamine adsorption on Amberlite IRC-50.

an ion exchanger column measuring 10 by 60 mm is shown in Table I. — The margin for the permissible salt content on a column of this size was regarded as quite safe for normal urine. Hence no measures to reduce the salt content, *e. g.* by precipitation with acetone, were necessary in the determination of free histamine.

3. Table II shows the *recovered amount of histamine added to urine.* The yield ranged from 70 to 95 per cent, mean 77 per cent. An adsorption of this order of magnitude was obtained when the drip rate was kept below 5 ml per minute.

4. *Adsorption of histamine by stirring the Amberlite in the histamine solution.* — With a view to simplifying the method described above, the following procedure was adopted. To 100 ml of an aqueous solution of histamine (pH 6.5) was added 5 ml of Amberlite IRC-50 the mixture then being

stirred for a few minutes with an electromagnetic stirrer. The water was discarded and the adsorbed histamine eluted from the ion exchanger by 0.1 N HCl. This method gave a recovery of 80—95 per cent from a water solution. The yield is, however, lower on increasing salt concentration. Hence the method was not useful for routine use in histamine analyses in urine.

Evidence that the extracted substance is histamine

Urinary extracts obtained with the procedure described above were tested on isolated guinea-pig ileum and rat colon and in a few cases the action was also studied on cat's blood pressure. Routinely a typical histamine response was obtained on guinea-pig ileum, but in no case was any effect observed on rat colon. As regards the effect on cat's blood pressure, a transient fall was noted. The effects on guinea-pig ileum and cat's blood pressure were totally blocked by Ergitin but were not influenced by atropine. With a histamine preparation extracted and purified by Arvidsson, Pernow & Swedin (1956) we obtained total breakdown of the biologically active factor.

The presence of histamine in the urinary extracts was demonstrated by paper chromatography. The solvent used here consisted of butanol/acetic acid/water (40 : 10 : 50), and diazotized sulfanilic acid was employed for developing.

Histamine excretion in human subjects

The material consisted of 17 healthy medical students — 10 males and 7 females — aged from 22 to 30 years. In 9 cases the urine was collected for 24 hours. From 8 of the subjects the urine was collected during two 24 hours periods. The urine was divided in a day (0700—1900 hours) and a night (1900—0700 hours) sample.

The figures obtained for the excretion of free and conjugated histamine are given in Table III. Since no significant difference existed between male and female excretion values, all figures are given in the same table. The statistical analysis is given in Table IV. The results given in this table show that the excretion of histamine by day is significantly higher than by night. No difference was obtained between the corresponding figures for the conjugated histamine.

No correlation was obtained between the urinary excretion of histamine (free and conjugated) and the 24 hour diuresis (Figs. 2—4).

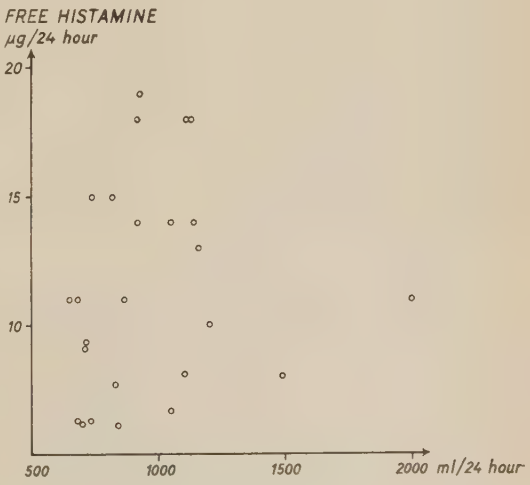

Fig. 2. Correlation between excretion of free histamine in urine and the diuresis.

Table III. *Excretion of free and conjugated histamine in 17 healthy subjects aged 22—30 years.*

Subject	Day (0700—1900 hours)		Night (1900—0700 hours)		Day+Night		Amount of urine in 24 hours
	Free histamine µg	Conjugated histamine µg	Free histamine µg	Conjugated histamine µg	Free histamine µg	Conjugated histamine µg	
1 a	4.3	3.0	2.0	1.0	6.3	4.0	730
1 b	5.4	1.0	4.0	1.3	9.4	2.3	715
2 a	10	27	3.7	15	14	42	1140
2 b	11	6.7	7.6	10	19	17	930
3 a	5.7	12	2.3	20	8.0	32	1490
3 b	8.5	25	3.3	23	11	48	2000
4 a	8.2	6.4	3.0	4.7	11	11	650
4 b	4.7	6.0	2.0	7.3	6.7	13	1050
5 a	6.2	10.1	3.0	3.8	9.2	14	710
5 b	6.0	6.7	5.3	4.0	11	11	680
6	5.7	32	2.0	24	7.7	56	830
7	5.3	14	1.0	4.2	6.3	18	680
8	7.7	17	10	30	18	47	1120
9 a	7.4	3.3	5.2	2.3	13	5.6	1160
9 b	3.3	6.8	2.8	5.0	6.1	12	840
10	9.2	27	4.9	24	14	51	920
11	6.0	4.3	2.1	5.0	8.1	9.3	1100
12	11	26	4.0	12	15	38	740
13 a	6.2	9.0	3.7	8.0	10	17	1200
13 b	11	9.0	7.2	11	18	20	920
14 a	7.0	12	4.0	10	11	22	865
14 b	3.9	7.1	2.3	8.0	6.2	15	700
15	7.0	5.3	8.0	9.0	15	14	815
16	12	10	6.0	7.3	18	18	1115
17	8.5	3.0	6.2	11	14	14	1050
Mean	6.9±0.5	11.6±1.8	4.22±0.5	10.5±1.6	11.9±0.8	22.0±3.14	
Range	3.3—10	1.0—32	1.0—10	1.0—30	6.1—19	2.3—56	

Table IV. *Statistical analysis of Figures given in Table III.*

Differences: (Significance tested by Student's t-test).

Day:	Bound—Free:	4.74±1.85,	t=2.56	fg=48	P= 0.01
Night:	Bound—Free:	6.24±1.65,	t=3.84	fg=48	P= 0.001
Day—Night:	Free:	2.63±0.6650,	t=3.95	fg=48	P=<0.001
	Bound:	1.13±2.39,	t=0.47	fg=48	P=<0.5

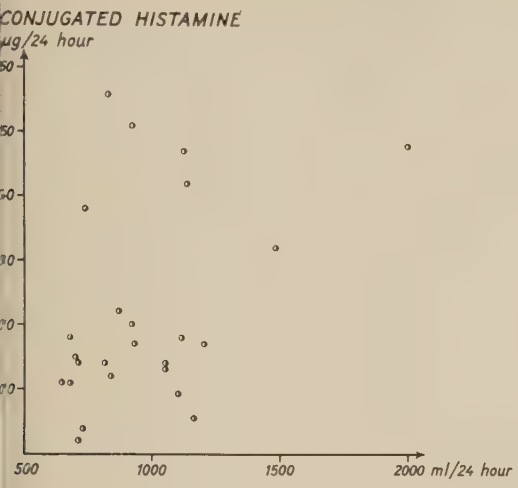

Fig. 3. Correlation between excretion of conjugated histamine in urine and the diuresis.

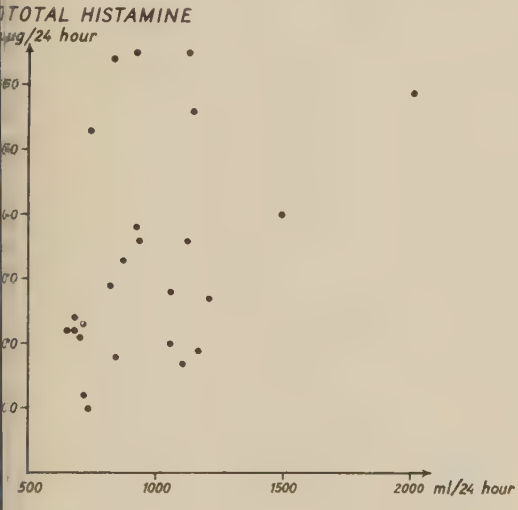

Fig. 4. Correlation between excretion of total histamine in urine and the diuresis.

DISCUSSION

It is essential for the determination of the histamine content of urine, that the urinary samples be collected in the proper manner. It is well known that histamine may be formed in urine from histidine in the presence of decarboxylating bacteria. The values obtained for the excretion of histamine *via* the kidneys will then be erroneous. In a few cases we have observed doubling of the histamine content of infected urine from women after storage at room temperature for six hours. Addition of histidine to such urine will rapidly result in a substantial elevation of the free histamine (von Euler, 1955). Acidification of the urine immediately after micturition will eliminate the danger of histamine formation. A pH level below 4 should constitute an adequate safeguard.

Probably there is little risk that histamine is inactivated by histaminase eventually occurring in urine. However, acidification of the urine to a pH level below 4 will also eliminate this possibility of an alteration in the histamine content.

The values for urinary histamine in healthy subjects obtained with the method described here are closely consistent with the normal values for free histamine reported by Adam (1954). The formerly employed animal charcoal method has either yielded substantially lower values (Anrep *et al.*, 1944), or has quite failed to show any free histamine in normal persons (Ungar & Pocoulé, 1937; Kappeler-Adler, 1941).

In our series we found, in accordance with earlier observations (Anrep *et al.*, 1944), that the urinary excretion of histamine was independent of the diuresis.

In most cases the greater part of the total 24-hour excretion consisted of conjugated

histamine. The excretion of conjugated histamine is on the whole constant day and night (means 11.6 ± 1.8 and 10.5 ± 1.6 μg respectively). Moreover, as shown by Anrep *et al.* (1944), it is largely dependent on the diet, the highest values coinciding with high protein intakes. The comparatively small range of variation of the values in this series probably indicates, therefore, that the subjects had fairly uniform diets.

The excretion of free histamine, on the other hand, showed a statistically significant difference between day and night means: (6.9 ± 0.5 and 4.2 ± 0.5 μg respectively). The higher diurnal excretion may conceivably reflect a greater endogenous production by day. Histamine administered orally is excreted, as shown by Urbach (1949) and Adam (1950), entirely in conjugated form. The significance of this endogenous physiologic histamine production still remains to be elucidated.

SUMMARY

1. A method, modified after Bergström & Hanson (1953), for determining free and conjugated histamine in urine is described.

2. Twenty-five determinations of histamine in day and night urine were carried out in 17 healthy subjects.

3. No correlation was found between the urinary volumes and the amounts of histamine excreted.

4. Free histamine varied in the day urine between 12 and 3.3 μg (mean 6.9 ± 0.5 μg), and in the night urine between 10 and 1.0 μg (mean 4.2 ± 0.5 μg). The corresponding values for conjugated histamine were 32 — 1.0 μg (mean 11.6 ± 1.8 μg) by day and 30 — 1.0 μg (mean 10.5 ± 1.6 μg) by night.

5. No significant difference existed between the amounts of conjugated histamine in the day and the night urine. On the other hand, the diurnal excretion of free histamine was significantly higher than the nocturnal excretion. This higher diurnal output may conceivably reflect a greater endogenous production by day than by night.

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SERUM PROTEINS AND FIBRINOLYSIS IN POLYCYTHEMIA VERA

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In an excellent monograph Lawrence (1955) gave a survey of the results which his team had obtained during the last two decades in their investigation of the pathophysiology, therapy and prognosis of polycythemia vera. Though the nature of the primary metabolic disturbance is not understood, fairly recent observations have clarified some of the mechanisms of intermediate metabolism in polycythemia vera. Thus, Huff, Elminger, Garcia, Cockell & Lawrence (1951) found the turnover of the serum iron to be several times greater than that suggested by the total red blood cell volume, supposing the life span of the red cells to be normal. Berlin, Lawrence & Lee (1951), who used the method of London, Schemin, West & Rittenberg (1949) for determining the life span of the red cells with the help of N^{15}-glycine, obtained results suggesting the formation of two different populations of red cells in untreated polycythemia vera, one with a normal, the other with a short life span. Lawrence expressed the view that the extremely high turn-over rate of the blood cells with a short life span can help to explain the abnormally rapid serum iron turnover in untreated polycythemia vera. In patients with secondary polycythemia Huff *et al.* found no evidence suggesting the formation of two such populations.

Thus in contradistinction to secondary polycythemia, polycythemia vera is characterised by an abnormally rapid serum iron turnover suggesting a hemolytic trait in the picture of the disease. Recently, v. Studnitz (1956) found that the alpha-2-globulins are regularly decreased not only in liver cirrhosis but also in pernicious anemia and in various hemolytic anemias. This type of change is rarely seen in other diseases. This prompted us to investigate whether the alpha-globulins showed the same changes in polycythemia vera as in the hemolytic anemias.

The fibrinogen content and the fibrinolytic activity of plasma were also determined, because one of us (I.-M. N.) had observed spontaneous dissolution of the blood clots from 2 patients with polycythemia vera when the blood was stored at room temperature for 24 hours. Lysine ethyl ester (LEe) was added to the blood sample on collection in order to prevent false low fibrinogen values as a consequence of fibrinogenolysis by active plasmin. Plasmin has a stronger affinity for LEe than for fibrinogen and is therefore useful as a plasmin inhibitor in short-term experiments. Not until all the LEe added is broken down by the plasmin, is the fibrinogen attacked (Troll, Sherry & Wachman, 1954).

MATERIAL

The material consisted of 19 patients with polycythemia vera and 3 with secondary polycythemia. Of the 19 patients, 4 had never received any treatment, the others had been treated with P^{32} for varying periods. The blood samples were collected when the patients presented themselves at the hospital for a new dose of P^{32}, in other words, all of these 19 were in a more or less pronounced relapse.

METHODS

Fibrinogen was determined by the method of Jacobsson (1955). 0.1 ml of lysin ethyl ester (LEe) (10 per cent) was added per 10 ml of blood collected.

Modification: the clot was dissolved in NaOH (0.1 N) at 100° for 10 minutes instead of in urea.

Prothrombin. — The prothrombin was determined according to Owrens P & P method (Owren & Aas, 1951).

Fibrinolysis. — Plasmin activity was determined in citrated plasma according to the method of Astrup & Müllertz (1952). The fibrinolytic activity was also roughly assessed by placing clotted blood at 37° and noting the time necessary for the clot to undergo lysis. Corresponding tests were further carried out on recalcified citrated plasma in varying dilutions. The lytic activity was compared with that of a crystalline trypsin standard.

Electrophoretic protein fractions were determined according to Laurell, Laurell & Skoog (1956) and *Polysaccharide* fractions according to Laurell & Skoog (1956).

RESULTS

The results are given below in tabular form. The mean value and the standard deviation are given for every plasma component studied. Standard normal values as determined in this laboratory are given when considered necessary for the evaluation of the results. The data in Table I suggest the degree of hematological disorder at the time of the investigation.

The serum proteins were found to be normal (Table II) with the exception of the γ-fraction, which varied between normal and distinctly pathologic (0.66 lowest value and 1.81 highest).

It is clear from Table III that the *polysaccharide* content was subnormal in the α_2-fraction and somewhat increased in the albumin and γ-fractions. The value for the *prothrombin* including so-called proconvertin was normal ($M = 84$, S.D. $\pm$ 12); the one-stage prothrombin time was also normal, which militates against the assumption that there is any appreciable lack of factor V. All of the cases showed active fibrinolysis. The mean value of the *plasmin* activity corresponded to 32 (S.D. $\pm$ 17) micrograms of trypsin per ml of 10 per cent citrated plasma. The blood clots obtained dissolved within 8—24 hours on incubation at 37° C. The lysis times of clots formed on 10 per cent citrated plasma was 6—12 hours. The lysis times of 10 per cent plasma clots from normal donors were 48—72 hours.

No correlation was found between the degree of abnormality of any of the variables studied. The following correlations were studied: fibrinolysis—fibrinogen content; number of erythrocytes—fibrinolysis; α_2-glycoprotein concentration—fibrinolysis; α_2-glycoprotein concentration — number of erythrocytes; γ-fraction — number of erythrocytes. Sera from 3 cases of *secondary polycythemia* showed slight hyper-γ-globulinemia (2 cases) and all of them showed an increase of the polysaccharide content of the albumin and the γ-fraction and a decrease in the polysaccharide content of the α_2-fraction. The changes were of the same magnitude as in polycythemia vera. However, none of these bloods showed fibrinolytic activity in any of the tests.

Table I. *Variations of the blood cells in the 19 cases of p.v.*

	Hemoglobin g per 100 ml	Red cells mill. per cmm	Leukocytes thousand per cmm	Thrombocytes thousand per cmm
Mean value	18.7	6.1	10.4	300
S.D. (σ)	1.9	0.8	5	120

Table II. *Plasma proteins in the 19 cases of p.v. The normal values (85 cases) are given in brackets. All values are expressed as g per 100 ml serum.*

	Serum protein	Albumin	α_1	α_2	β_1	β_2Ca	γ	Fibrinogen
Mean value	7.5 (7.2)	4.8 (4.61)	0.33 (0.34)	0.47 (0.51)	0.50 (0.50)	0.25 (0.33)	1.10 (0.88)	0.33 (0.30)
S.D. (σ)	0.5 (0.26)	0.3 (0.20)	0.03 (0.037)	0.07 (0.056)	0.07 (0.05)	0.06 (0.045)	0.29 (0.12)	0.06 (0.02)

Table III. *The electrophoretic distribution of the protein bound hexoses in serum from 19 patients with p.v. The normal values (54 cases) are given in brackets. All values are expressed as mg hexose per 100 ml serum.*

	Total hexose	Albumin	α_1	α_2	β	γ
Mean value	123 (120)	19 (10.2)	21 (25)	27 (42)	28 (31)	23 (11)
S.D. (σ)	16 (5.7)	4 (2.5)	4 (3.2)	6 (4.8)	5 (4.3)	6 (3.8)

DISCUSSION

The results showed that the same type of changes occurred in the untreated patients with p.v. as in those treated but during relapse. Therefore, the material was treated statistically as a single group.

Serum proteins. — In half of the cases the gamma globulins were found to be increased. The reason for this is unknown.

The mean value found for the alpha-2-globulins did not differ statistically from the normal. Only 3 cases showed clearcut subnormal values. The decrease in the alpha-fraction is thus not so marked in polycythemia vera as it is in the hemolytic anemias or pernicious anemia.

Glycoproteins. — As yet no systematic investigations are available of the electro-

phoretic glycoprotein spectra in various diseases. The biologic function of these fractions is still obscure. Therefore the observations made will not be discussed but simply described.

The carbohydrate content of the alpha-2-fraction was regularly decreased in all of the patients, while the protein concentration of this fraction was subnormal in only 3. The relative carbohydrate content of the alpha-2- fraction was thus definitely subnormal. This shows that in polycythemia vera the concentration of one or more of the carbohydrate-rich components of the alpha-2-fraction is decreased. One of the components richest in carbohydrates and serving as a carrier of hemoglobin in the plasma is haptoglobin (Jayle, 1954). We have not yet determined the haptoglobin concentration in polycythemia vera but we know that it is decreased in hemolytic anemias (Nyman, 1956). Since polycythemia vera is a disorder with a hemolytic trait, it appears that at least part of the decrease in the α_2-fraction might be due to a decreased concentration of the haptoglobin in the plasma.

Both the relative and the absolute carbohydrate content of the gammafraction were somewhat increased.

It was noteworthy that all of the blood samples from polycythemia vera studied, showed fibrinolytic activity, but that under the experimental conditions used all of the normal blood samples were without fibrinolytic activity, as were those from 3 patients with secondary polycythemia. This might help to explain "that a paradoxical situation is present in patients with p.v. in that there is both a high incidence of hemorrhage from various vessels — and also a high incidence of thromboses" (Lawrence, 1955). The tendency to bleeding might be due to the occurrence of active proteolytic enzyme in the plasma and the tendency for thromboses may *inter alia* depend on the high blood viscosity.

The analyses hitherto carried out only showed that on dilution polycythemia vera plasma contained a markedly increased fibrinolytic activity. Investigations are in progress to determine which component or components in the fibrinolytic system are responsible for this increase. Several possibilities may be considered. Changes might occur in the concentration of the plasminogen, the proactivator of plasminogen, and/or of the plasmin inhibitors. In this connection it might be mentioned that the strongest plasmin inhibitor migrates electrophoretically with the α_2-globulins and that we found the carbohydrate content of the α_2 in these patients to be subnormal.

We could not confirm the findings of earlier investigators (Björkman, 1947; Fiehrer, 1950; Stefanini & Dameshek, 1955) that the fibrinogen concentration is sometimes decreased in polycythemia vera. Their observations can be explained as a methodological artefact since these authors did not inhibit the active fibrinolytic plasma factor during the blood sampling and analysis of the fibrinogen content.

It is remarkable that, in contradistinction to the patients with secondary polycythemia, all of the 19 with polycythemia vera showed spontaneous fibrinolytic activity. Further investigations are necessary to determine whether measurement of the fibrinolytic activity of the plasma can be used in the differentiation between polycythemia vera and secondary polycythemia.

SUMMARY

Analysis of the plasma proteins in polycythemia vera suggested the following conclusions:

1) The polysaccharide content in the electrophoretic α_2-fraction is decreased in polycythemia vera and in secondary polycythemia,

2) The fibrinogen content is normal in polycythemia vera and secondary polycythemia,

3) Plasma from 19 patients with polycythemia vera contain an active fibrinolytic factor. In contrast, plasma from 3 patients with secondary polycythemia showed no fibrinolytic activity.

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RENAL FUNCTION STUDIES IN DIABETIC NEPHROPATHY[1]

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Many publications have stressed the progressive nature of the vascular complications found in diabetes mellitus. The complications have been considered to be the result of an unphysiological control of the diabetic patient, *i. e.* the patients have been permitted to have an elevation of the blood sugar and varying amounts of glycosuria.

Larsson, Lichtenstein & Ploman among others (1952) have objected to this conclusion. They permit a limited increase of the blood sugar and a moderate glycosuria in their patients. They consider the vascular complications in their patients to be of a similar frequency as those found in diabetics where treatment has been of a more restricted type.

The unknown cause of the high frequence of vascular complications in late diabetes and the dominant role which the renal complications play (Joslin, 1950) as cause of death, has stressed the importance of intensified research in this field. Or, as Joslin has put it, "A constant awareness for evidences of incipient signs of renal disease must be maintained. Research should be concentrated on the kidney in the young diabetic."

Among the earlier renal function studies in diabetics Hogeman's (1948) work should be mentioned. He found that the inulin clearance was lowered more than the diodrast clearance in patients with diabetic nephropathy. Hilden (1949) has shown that the clearance of sodium thiosulphate and the value for diodrast T_m, calculated with the aid of the thiosulphate clearance, was lowered to the same degree.

Robertson, Gray & Baynes (1951) have determined the clearance for inulin and para-amino-hippurate and the T_m for para-amino-hippurate and glucose (on venous blood) in nine patients with the syndrome of Kimmelstiel—Wilson. In Robertson's material the clearances for inulin and para-amino-hippurate were lowered to the same extent as the T_m of para-amino-hippurate and glucose. They considered that a more detailed study might throw light on the mode of development of the lesions. The necessity for further studies of renal function in diabetes is clearly indicated in the publication of Brun, Gormsen, Hilden, Iversen & Raaschoug (1953), on the basis of renal biopsies obtained in patients with this disease. From their work, it is obvious that patients with diabetic nephropathy may have some glomerular function preserved in spite of severe patho-

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Table I. *Clinical findings in the cases of diabetic glomerulosclerosis. d.r. = diabetic retinopathy.*

Case No.	Age	Sex	BSA	Duration in years	Blood pressure	Eyegrounds
3	40	♀	1.70	17	160/90	III+d.r.
6	26	♀	1.63	19	150/120	III+d.r.
8	20	♀	1.65	11	125/100	d.r.
10	26	♀	1.76	12	125/50	III+d.r.
15	25	♀	1.34	15	210/105	IV+d.r.
16	31	♀	1.58	7	145/100	d.r.
17	35	♀	1.59	24	150/100	d.r.
18	31	♀	1.65	18	190/140	I+d.r.
19	29	♂	1.56	20	145/90	I+d.r.
20	24	♀	1.68	17	130/105	d.r.

anatomical changes of the nephron. This agrees with the findings obtained from renal biopsy studies in glomerular nephritis (Parrish, Rubenstein & Howe, 1955).

The present investigation is an attempt to analyse the nature of the renal lesions in cases of diabetes with varying degrees of renal damage. Inulin clearance, endogenous creatinine clearance, para-amino-hippurate clearance, para-amino-hippurate and glucose T_ms have been determined in all the patients. The value of the T_m in estimating renal function in physiological states is not beyond question. In patients with renal lesions it is apparent that the results of T_m determinations must be judged with even more caution. Nevertheless glucose and para-amino-hippurate T_m have been determined in this investigation because it was hoped that they might give some information concerning the nature and the localisation of the renal lesions.

The renal extractions of para-amino-hippurate have been determined by renal vein catheterization in all patients in this series.

Although the T_m determination is considered to be a concept of limited value, technical faults must be avoided in its determination. It is incorrect to use the thiosulphate clearance as a measure of the glomerular filtration rate. Sodium thiosulphate, as has been shown earlier (Bucht, 1949) can be both reabsorbed and excreted by the tubules. In glucose T_m determinations it is essential to use arterial rather than venous blood.

The patients have not been chosen at random. On the contrary the aim has been to study, as far as possible, pure diabetic lesions, of varying degrees of severity. The material consists of 10 patients, who, at the time of investigation, were under the care of the Fourth Medical Department at St. Erik's Hospital. Some of the clinical findings are illustrated in Table I.

As we wished to study pure diabetic nephropathy, we have chosen young patients. This makes the clearance and extraction determinations easier from a technical point of view. In all the patients, except one, the disease was of more than 10 years duration. All, except one, had hypertension. All had retinal changes. It is remarkable that many of the patients had advanced hypertensive

retinal changes in spite of a slight elevation in their blood pressure.

A more extensive analysis of the relation between clinical findings and the renal lesions in this group of patients has been published earlier (Bucht, Werkö & Ek, 1953). This study showed that the clinical findings are similar to those usually found in diabetes, and that the severity of the vascular changes was correlated to the duration of the disease.

Since the conclusion of this investigation, 3 of the patients have died in uremia. Postmortem microscopic examination was only obtained in one patient (No. 15). The picture was that of pure diabetic nephropathy.

METHODS

All patients were investigated in the morning, and were in the post absorbtive state, except that they had one litre of water to drink about one hour prior to the study. They did not get their usual morning dose of insulin. Para-amino-hippurate was given intramuscularly (according to Bucht, 1951) to obtain a low, slowly decreasing plasma concentration of para-amino-hippurate. After that, the blood concentrations of para-amino-hippurate were increased, stepwise, by means of an intravenous sustaining infusion in a manner described earlier (Josephson, Bucht, Ek & Werkö, 1952). Inulin was given intravenously in one single injection (according to Alving & Miller, 1943). This method of giving inulin is far from ideal, but it may be used in cases like these were variations in the filtration rate or diuresis are not to be expected during the course of the investigation.

The methods for analyses are published earlier (Bucht, 1951). These methods have been followed, except that plasma and urine were yeasted. The blood sugar has been determined according to Folin and urine sugar according to Benedict. The blood specimens were taken from an indwelling arterial needle in one of the brachial arteries. The renal vein blood was obtained from a catheter in the renal vein. The urine was obtained from an indwelling catheter. The bladder was washed out twice with distilled water and finally with air.

The creatinine was determined according to Folin, modified for a photoelectric colorimeter.

The endogenous creatinine clearance has been determined partly as a check for the completeness of bladder emptying, and partly because an earlier observation showed that the quotient inulin clearance / endogenous creatinine clearance often was found to be low in diabetic patients, despite the absence of signs of renal failure.

CALCULATIONS

The clearance values were calculated in the usual way. The para-amino-hippurate extraction was calculated according to the formula

$$E = \frac{(Ac - Vc)100}{Ac}$$

E stands for the renal extraction of para-amino-hippurate in per cent; Ac for the arterial concentration; Vc for the venous concentration of para-amino-hippurate.

The mean for the normal extraction values in our hands is 90.6. Para-amino-hippurate and glucose T_m have been calculated in the usual way (Homer Smith, 1951). The tubular load has been calculated according to the formula

$$\text{Load} = AC \times RPF - 0.8\,F \times Cin \times Ac.$$

RPF = Renal plasma flow; Cin = Inulin clearance.

The values for the inulin clearance are averaged from all periods; usually 8 to 10 periods, of about 15 minutes duration. The value for the renal plasma flow is calculated from the first two periods with low plasma concentration.

The filtration (ff) in his material has been calculated from the inulin clearance and the renal plasma flow, and not from the para-amino-hippurate clearance, as is customary.

RESULTS

Table II shows the results primarily of the clearance studies. It can be seen from Tables I and II that the material consist of patients

Table II. *Renal plasma flow, clearance for inulin and creatinine and T_m for glucose and para-amino-hippurate in ten cases with diabetes mellitus all values corrected to a body surface area of 1.73 m².*

Case No.	Proteinuria per thousand	NPN	Basal PAH conc. mg per cent	Inulin clearance ml/min. /1.73 m²	Endogeneous creatinine clearance ml/min.	Renal plasma flow ml/min. /1.73 m²	ff	Creatinine clearance / Inulin clearance	PAH Extraction per cent	PAH Tm mg/min. /1.73 m²	Glucose Tm mg/min. /1.73 m²
3	1.5—6	83	2.01	21	26	345	6.2	1.24	40	9	69
6	2—3	34	1.18	56	51	342	16.4	0.91	80	45	131
8	0.5—1.5	30	2.95	122	—	705	17.4	—	90	69	422
10	0.5—3	26	0.84	101	61	465	21.7	0.60	39	97	228
15	2—4	78	3.00	11	15	252	4.5	1.36	25	5	25
16	0	32	1.79	123	42	689	17.8	0.42	93	73	270
17	0.1	38	1.71	109	44	621	17.5	0.40	90	(40)*	302
18	3—5	36	1.22	64	47	495	12.9	0.73	81	52	188
19	0.1—12	47	2.31	40	35	300	13.4	0.88	74	26	129
20	0.5—1	28	0.31	46	37	355	13.0	0.80	85	23	208

* Probably not above saturation — limit.

with diabetic vascular damage of varying severity. The values for inulin clearance varied between 11 and 123 ml/min. The renal plasma flow varied between 252 and 689 ml/min. The filtration fraction was low in all cases, in spite of the fact that almost all of the patients had hypertension.

The para-amino-hippurate extraction was calculated from the lowest value for the plasma concentration. It was lowered in seven cases, and normal in three. The values for para-amino-hippurate and glucose T_m vary to the same extent as do the inulin clearance for PAH 5—97 mg/min, and for glucose 25—422 mg/min.).

The endogenous creatinine clearance values are as a rule lower than inulin clearance values. The ratio between creatinine and inulin clearance varies between 0.40—1.36.

Fig. 1 shows the ratio between the renal extraction of para-amino-hippurate and inulin clearance in the diabetics and in a group of patients with hypertension and gomerulo-nephritis.

In the diabetics the para-amino-hippurate extraction is relatively well preserved at values for the inulin clearance of about 40—50 ml/min. Below this level it decreases abruptly. Some hypertensive patients, on the other hand, with almost normal values for inulin clearance showed moderate decrease of the para-amino-hippurate extraction. The extraction values in patients with nephritis show a larger spread than the values in the other groups.

Fig. 2 shows the ratio between the para-amino-hippurate extraction, and the para-amino-hippurate T_m. The results agree with those in Figure 1. From a practical point of view, one can anticipate a normal para-

amino-hippurate extraction with values for the para-amino-hippurate T_m of about 60 mg/min and more.

Fig. 3. The values for glucose T_m and para-amino-hippurate extraction, show a similar relation to that of para-amino-hippurate and inulin shown in Fig. 1. With values for the glucose T_m of above 200 mg/min. the para-amino-hippurate extraction can be considered to be normal.

Fig. 4 shows the ratio para-amino-hippurate-glucose T_m plotted against inulin clearance. The regression lines are drawn according to the least square method. The decrease of the T_{ms} seem to run parallel to the decrease of the inulin clearance.

Fig. 5 demonstrates the extraction of para-amino-hippurate at varying tubular loads of para-amino-hippurate. The para-amino-hippurate concentrations are noted in figures at the top of the curves at the spot where the extraction seems to decrease rapidly. Their position is meant to be an indication of the depression limit. From a practical point of view, it is of value to know the depression limit in normal, or relatively normal kidneys. With this information the concentration may be maintained at a level which will not cause self-depression of the para-amino-hippurate clearance. From the theoretical point of view, the concept of a limit is a false one, as it is not the limit but the load in units/min., which is of importance in the kidneys' ability to extract para-amino-hippurate from the arterial blood (Josephson, 1947). The concept "depression limit", furthermore, gives no practical help in assessing kidney damage (see below).

DISCUSSION

Inulin clearance may be used as a measure of the glomerular filtration rate in patients with diabetes, as it seems to be a sensitive measure of renal damage. Objections may be raised to its use as a valid measure of the glomerular filtration rate in all conditions. However, at the present, no better measure of the glomerular filtration rate can be obtained by any other method.

The clearance of endogenous creatinine as determined in this paper is not a good measure of the glomerular filtration rate in diabetics.

The clearance of endogenous creatinine is lower than the inulin clearance, except in cases 3 and 15. These patients had a markedly raised non-protein nitrogen in the blood, and also a raised plasma creatinine. These conditions tend to promote the excretion of creatinine.

The marked lowering of the clearance of endogenous creatinine, in comparison to the inulin clearance in some cases, is partly due to raised plasma glucose (Haugen, 1954), and partly to the presence of ketogenic substances in the blood. The ketogenes are known to give a false creatinine reaction with the alkaline picrate.

The cases which have an inulin clearance above 50 ml per minute, and a para-amino-hippurate and glucose T_m above 60 and 200 mg/min. respectively (see Figs. 1 and 4), can be considered to have a normal or near normal renal extraction of para-amino-hippurate.

Fig. 1 demonstrates that there are some differences in the relationship between the extraction of para-amino-hippurate and inulin clearance in the diabetics as compared

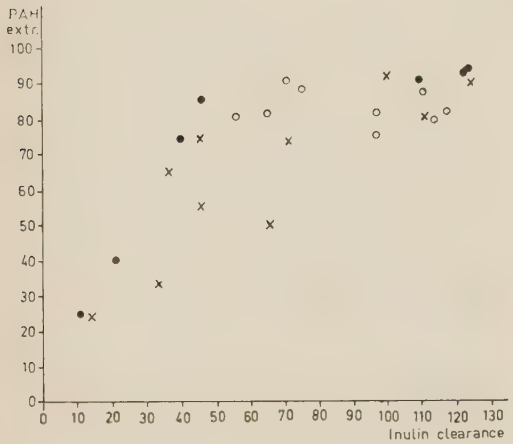

Fig. 1. Renal PAH-extraction in cases with hypertension (o), nephritis x and diabetes (●) plotted against inulin clearance.

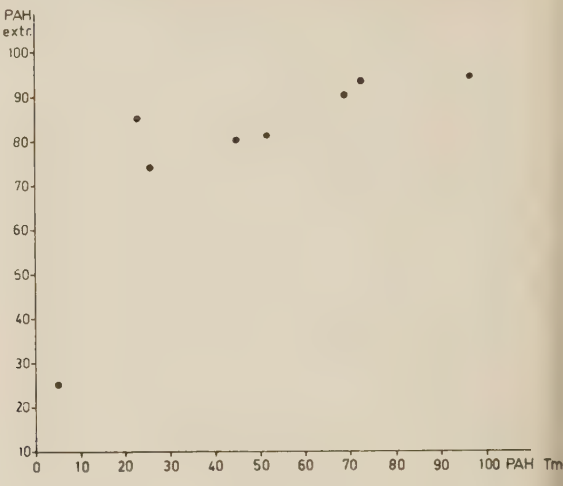

Fig. 2. Renal extraction of PAH plotted against PAH Tm.

to the patients with glomerular-nephritis and hypertension, at low concentrations of para-amino-hippurate in the blood. The hypertensive patients, as pointed out above, may have a slightly lowered para-amino-hippurate extraction, in spite of a well preserved inulin clearance.

The patients with diabetes and those with nephritis may differ in one respect. The diabetics seem to have a straight line correlation between their renal extraction of para-amino-hippurate and the inulin clearance. But, on the other hand, they show a wider spread of the values. The difference in behavior of the two groups is too small to permit any definite conclusions. It may merely be that the nephritic group is clinically a more heterogenous one. A patient with acute nephritis who has an inulin clearance of the same magnitude as one with a chronic form of the disease, will not invariably behave in the same way in regard to the extraction of para-amino-hippurate at low loads.

In the diabetic patients, who were as free from complications as possible, a relatively well preserved para-amino-hippurate extraction was found even when there was a markedly lowered inulin clearance.

The tubular function, as measured by T_m, is lowered to the same extent as the inulin clearance (see Fig. 4). It had been supposed earlier that the decreasing amount of glucose in the urine in advanced cases of diabetic nephropathy would depend on a glomerulotubular imbalance, *i. e.* a more severe destruction of glomeruli than of the tubules. Such a glomerulo-tubular imbalance is not present in this group of patients.

Fig. 4 shows the ratio between para-amino-hippurate and glucose T_m and inulin clearance. The ability of the tubules to

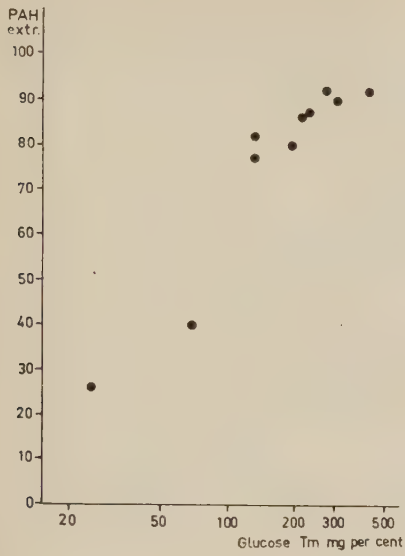

Fig. 3. Renal extraction of PAH plotted against glucose Tm.

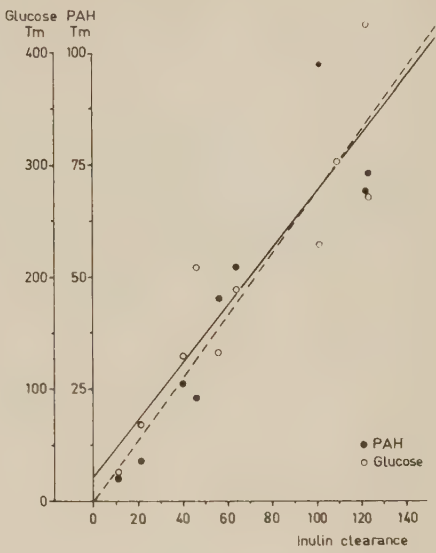

Fig. 4. PAH-Tm and glucose Tm plotted against inulin clearance.

excrete para-amino-hippurate and reabsorb glucose respectively are decreased to the same extent as is the inulin clearance. In this respect, the results coincide with those of (Hilden, 1943). Fig. 2 and 3 demonstrate the fact that the tubular function, in these cases measured by the ability to absorb glucose and excrete para-amino-hippurate, may be obviously decreased in spite of a good ability to clear the blood of para-amino-hippurate at low tubular loads. This suggests that the defect in the function of the nephron may be due to an imbalance in the function of the proximal and distal tubules.

Some of Oliver's (1949) microdissection investigations indicate that the morpho-biological changes in diabetes may be localized to the proximal tubules. Oliver's explanation is that the proximal tubules normally are exposed to a certain amount of glucose in the tubular urine while the distal part of the tubules are not, except in diabetes. Thus, the lesion may be an effect of hyperosmolarity. If this is the cause of the tubular lesion in diabetes, therapeutic efforts should be directed toward the maintenance of blood sugar within normal limits.

Fig. 5 demonstrates some further points of interest. In cases with a normal or a close to normal inulin clearance, we also have a normal para-amino-hippurate extraction, even at relatively high loads. All cases with a lowered inulin clearance show, on the contrary, a decreasing extraction of para-amino-hippurate with an increasing load, even at very low loads. This shows that even though the load is low, it exceeds the level necessary for complete extraction of para-amino-hippurate from the blood. The expression effective renal plasma flow is a common one, meant to give an estimate of the renal plasma flow in pathological cases

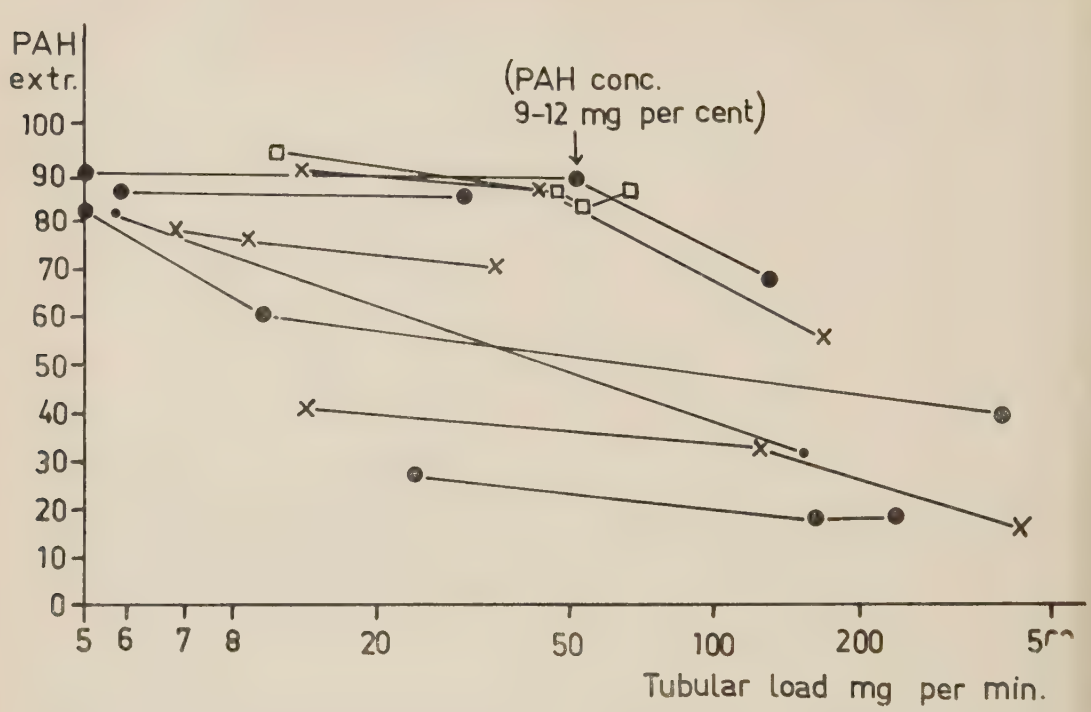

Fig. 5. Renal extraction of PAH plotted against tubular load of PAH.

where the renal vein is not catheterized. The term is based on the erroneous assumption that the para-amino-hippurate extraction, although low, should be constant at low plasma concentrations of para-amino-hippurate. This is not the case in diabetes, or in glomerulo-nephritis (Josephson *et al.*, 1952). The appearance of the curves in Fig. 5 suggests that the "depression load" has been exceeded, despite a low load of para-amino-hippurate. The limit for complete extraction may be even lower, and may approach zero as in cases with severe nephritis or hypertension (Josephson *et al.*, 1952). The expression "effective renal plasma flow" should be abandoned, at least in cases where the renal function is impaired.

The nature of the tubular damage in diabetic nephropathy can, from a functional point of view, be divided in two groups. In the one the tubules have a decreased ability to extract para-amino-hippurate, while in the other the nephrons are completely destroyed. As a result the blood would pass through inert nephrons, which should result in a lowered para-amino-hippurate extraction independent of the amount of para-amino-hippurate in the arterial blood. In this respect, the findings do agree with those obtained in glomerulo-nephritis. Even in other respects, the findings in nephritis and diabetic nephropathy agree (see Fig. 1). Furthermore, the two conditions both have

a low filtration fraction, in spite of their high blood pressure. In essential hypertension a high filtration fraction is found.

SUMMARY

1. A group of ten patients with diabetes mellitus and vascular lesions of differing severity were studied with renal vein catheterization. The renal extraction of PAH (at low PAH concentrations) and the renal plasma flow were calculated. The inulin and endogenous creatinine clearance and T_m for PAH and glucose were also determined.

2. Certain problems associated with the use of clearance values and T_m determinations as absolute measures of discrete renal functions in patients with diabetes have been pointed out.

a) The endogenous creatinine clearance is of limited value in determining the glomerular filtration rate while inulin clearance is a good measure of renal function in diabetes.

b) T_m determinations for glucose and PAH give no further indication of the extent of the renal damage than the determination of the inulin clearance.

c) The PAH clearance can not be used as a measure of renal plasma flow without renal vein catheterization when renal function as measured by inulin clearance is markedly impaired (below 50 ml/min.). Above this value the PAH clearance is only a relative measure of the renal plasma flow up to normal values for inulin clearance.

"Effective renal plasma flow" should not be used as a relative measure of the renal plasma flow in diabetes because the PAH extraction varies with the tubular load.

3. The kidney in diabetic nephropathy consists, from a functional point of view, of two types of nephrons. 1) Nephrons which are completely destroyed and 2) Nephrons which are damaged but may be able to extract PAH more or less completely at low loads.

This investigation suggests that a specific lesion for diabetes may exist with its site distal to the proximal part of the proximal tubule.

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VARIATIONS IN THE SERUM GLYCOPROTEIN CONCENTRATION DURING ADMINISTRATION OF INSULIN

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The glycoprotein concentration in the serum of diabetics increases when symptoms of angiopathy develop (Bergman, Rifkin & Ross, 1953; Hjelm Nielsen & Poulsen, 1954; Törnblom & Nordström, 1954; Andreani & Gray, 1956). There was the possibility of a connection between blood sugar and glycoprotein metabolism and Jacobs (1949) found a striking parallelism between the blood sugar level and the glucosamine concentration in a "non heat coagulable" mucoprotein fraction in serum. Further, he found direct proportionality between the glucose and glucosamine concentrations during administration of insulin to a diabetic patient and concluded: "Insulin seems to exert a pronounced effect on the level of glucosamine found by the method described".

Jacobs' finding has not been confirmed. Stary, Bursa, Kaleoglu & Bilen (1950) have demonstrated, on the contrary, that there is an *increase* in the serum glycoprotein concentration during insulin administration. Their experiment was carried out on schizophrenics with a normal carbohydrate metabolism. Stary concludes that the hyperglycoproteinemia is due merely to the dessication which results from perspiring excessively during insulin shock, as the polysaccharide/total protein ratio is not altered significantly.

The following investigation confirms Stary's finding. The glycoprotein variations have been investigated in the serum of three psychotic patients with normal carbohydrate metabolism during insulin coma therapy, and in one diabetic patient during insulin therapy for diabetic precoma.

METHOD AND RESULTS

In the psychotic patients the blood samples were taken fasting just before the insulin was administered, when the coma was deepest, and a couple of hours after the patient had awakened. Furthermore, 2—3 samples were taken on an empty stomach at intervals of several weeks during the period of treatment. The determinations made in the blood samples were total protein (tp) by the Biuret method, serum sugar (g) by Hagedorn-Jensen's method and glycoproteins expressed by the polysaccharide component (ps) which is equal to the sum of hexoses determined by the anthrone method (Graff, Greenspan, Lehman & Holechek, 1951) and glucosamine by the author's modification of Elson & Morgan's (1933) method (Rosenlund, 1956). The polysaccharide content of the total proteins is given in per cent (ps/tp · 100).

[1] The investigation was carried out with grants from the Norwegian Research Council for Science and the Humanities.

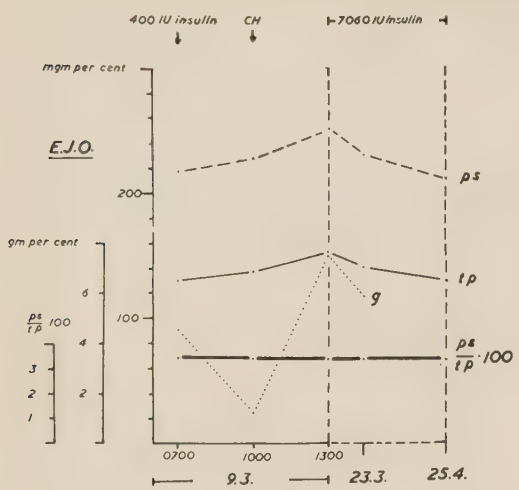

Fig. 1.

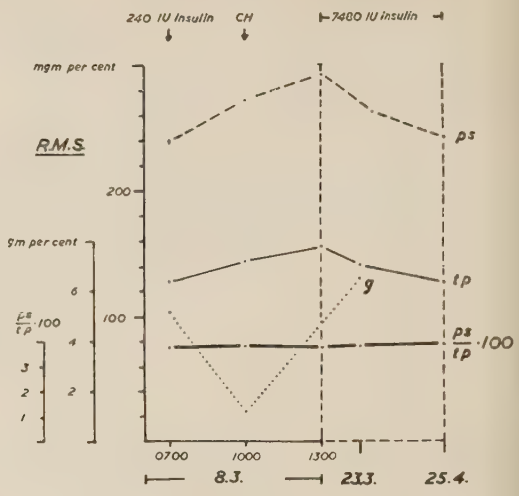

Fig. 2.

The results of these investigations are presented in curves in Figs. 1, 2 and 3. In all cases the ps/tp ratio was rather constant during the shock phase, which shows that the polysaccharide variations were only apparent and were due to alterations in the fluid balance. There is no proportionality between the blood sugar and the polysaccharide values. In two of the patients no significant changes occurred in the ps/pt ratio after several weeks' therapy. In the third patient there was some increase after a few weeks. This patient, however, had a myocardial injury, labile hypertonia and uterus prolapse in addition to her psychosis, and she reacted so violently to the therapy that it was discontinued on April 14. On April 22 she was operated for the prolapse which is probably the cause of the further increase on April 25, as it has been shown that an operation may lead to an acute increase of the serum glycoproteins. The glucosamine and hexose values varied parallel during the experimental period.

That Jacobs obtained results which showed parallelism between the glucosamine and blood sugar in the serum, as well as a reduction of the glucosamine values after administration of insulin, must be due to a disregard of alterations in the fluid balance. Diabetics with high blood sugar values will often be dehydrated with a consequently increased

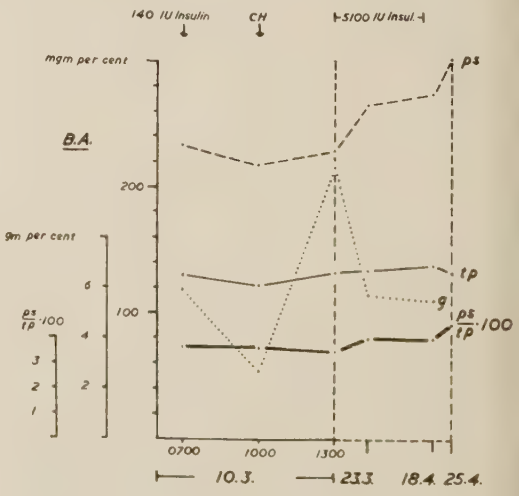

Fig. 3.

Table I. *Determinations in serum of glucose, glucosamine, total protein and glucosamine in per cent of total protein before and after insulin-fluid therapy. All of the values for the glucosamine/total protein ratio are within the range of normal variation*

	Glucose mg/100 ml	Glucosamine mg/100 ml	Total protein g/100 ml	Glucosamine in per cent of total protein
Before therapy	389	125	9.12	1.37
After therapy, days: 1	385	96	6.26	1.53
2	300	94	5.61	1.67
3	365	95	5.87	1.62
4	230	91	5.78	1.57

Table II. *Analyses of sera without and with addition of glucose.*

	Glucose mg/100 ml	Mucoproteinglucosamine by Jacobs' method. Optical density	Total glucosamine by the author's method. Optical density
Serum	93	0.172	0.291
Serum with glucose	312	0.410	0.284

ycoprotein concentration. During treatent with insulin and administration of uid the blood sugar values will decrease, ut at the same time the dehydration is iminated, whereupon the glycoprotein valps also decrease, causing parallel shifts of e blood sugar and glucosamine values. able I presents the data for a patient with previously untreated diabetes who was adtted in a precomatous condition. This ble illustrates the influence of therapy on e fluid balance. The glucosamine values hibit a pronounced decrease, while glucomine/total protein remains within the lim of normal variation the entire time (by is method $\bar{x} \pm 3$ S.D. $= 1.43 \pm 0.36$ per nt).

Furthermore, the method employed was less exact because the results are influenced by the glucose concentration. Immers & Vasseur (1952) point out that glucose together with certain amino acids will interfere with the reactions under certain conditions so that the glucosamine values will be too high. The prerequisites for this type of interference seem to be present in the method used by Jacobs as illustrated by the following experiment:

A serum containing 93 mg glucose/100 ml was divided into two portions and glucose was added to one of them to 312 mg/100 ml. Both sera were then analysed according to Jacobs' method and the author's.

method. The results (Table II) show that the serum to which glucose was added give more than double so high extinction values as in the original serum, while there is no significant difference in the extinctions when the author's method is employed.

SUMMARY

The possibility of a relationship between the serum glycoprotein level and the blood sugar concentration, as well as the influence of insulin on the serum glycoprotein metabolism is briefly discussed with reference to publications of Jacobs (1949) and Stary *et al.* (1950). Investigations on psychotic patients during insulin coma therapy confirm Stary's results as no definite relationship is found between glucose and glycoprotein values in serum under the influence of insulin. These experiments, as well as investigations of glucosamine variations in a diabetic patient during insulin-fluid therapy show that the glucosamine variations during administration of insulin depend solely on the alterations in the fluid balance. Further, Jacobs' method is less reliable because the "glucosamine values" determined by this method are influenced by the concentration of glucose in the serum analysed. Insulin can thus hardly influence the glycoprotein metabolism as postulated by Jacobs.

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SERUM FAT IN TOURNIQUET SHOCK

AN EXPERIMENTAL STUDY IN RABBITS, WITH SPECIAL REFERENCE TO THE ACTION OF HEPARIN[1]

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Studies on fat metabolism have during recent decades attracted an intense scientific interest. New methods such as the chemical fractionation technique, ultracentrifugation, zone electrophoresis of various kinds, and chromatography have stimulated these investigations. The new methods have given valuable information on the adsorption, transport, deposition, and mobilization of fat in normal and pathological states (Anfinsen, 1955).

Moore (1953) has shown that endogenous oxidation of fat is greatly increased after trauma. He found that after major injury about 12 per cent of the stored fat was used up owing to endogenous oxidation during the following 4—5 days.

Milch, Redmond & Calhoun (1954) have found changes in the lipo-protein pattern of rabbit serum following different kinds of trauma, the lipo-proteins being changed to higher Sf-classes. Following injection of heparin this change was found to be less.

The action of heparin on alimentary lipemia and its effect on the serum lipo-proteins have been studied by Herbst, Lever, Lyons & Hurley (1955), whose investigations have shown that 'clearing' of alimentary lipemia by means of heparin increases the electrophoretic mobility of the serum lipo-proteins.

In their investigations on fat-embolism Johnson & Svanborg (1956) found in rabbits a twofold increase in the total serum lipids 24 hours after trauma. Lipid-staining material was found in the pulmonary vessels. This was found to the same extent in rabbits that had been exposed to fractures as in rabbits that had been exposed to tourniquet shock.

In the present investigation the following questions have been examined by experiments on rabbits. 1. To what extent is the neutral-fat content of serum changed following trauma? 2. Is it possible, by means of injecting heparin, to influence the concentration of neutral-fat in serum after trauma? 3. Is the electrophoretic pattern of the serum lipo-proteins changed a) after trauma, b) after trauma + injection of heparin? 4. Can heparin prevent the appearance of fat in the lung capillaries of rabbits exposed to tourniquet shock? An investigation of the serum fat response to trauma in human subjects is in progress.

[1] This work has been supported by a grant from "Statens Medicinska Forskningsråd".

MATERIAL AND METHODS

20 male rabbits weighing 2300—3200 g (M. w. 2760 g) were used. The series was arranged as follows. *Group I,* 8 animals exposed to tourniquet shock. *Group II,* 8 animals exposed to tourniquet shock + heparin (1 mg/kg body weight intravenously every 6th hour). *Group III,* 4 controls.

Tourniquet shock

The animal had its last meal 18 hours before being exposed to trauma. An iron wire was tied tightly round one hind-limb, above the ischial tuberosity. Three hours later the wire was removed. During the experiment no food was given. A mixture of electrolytes (NaCl 5 g + $NaHCO_3$ 5 g + distilled water *ad* 1000) was freely supplied for drinking. Five ml of blood was aspirated from the heart before trauma, and 6, 24, and 48 hours afterwards. The blood was allowed to coagulate at room temperature, and aliquots of serum were then immediately taken for analysis. After the last sample (48 hours after the trauma) the animal was killed. Lung tissue was placed in formalin.

Extraction of lipids

1 ml serum was extracted with 20 ml of an ethanol ether mixture (3:1). After filtration the extract was transferred to a 25-ml volumetric flask, and made up to the mark. Aliquots of this extract were taken for the various determinations.

Determination of phospholipids

Lipid-P was determined after combustion with sulphuric acid in micro-Kjeldahl flasks, using perhydrol as oxidative agent. The factor of 25 was used for conversion of lipid-P to terms of phospholipids.

Determination of 'free glycerol' (FG)

An aliquot of the lipid extract was dried, and its content of FG determined by the method of Lambert & Neish (1950). (Glycerol is oxidized by per-iodic acid to formaldehyde, which is estimated colorimetrically after reaction with chromotropic acid.) The oxidation time with per-io lic acid was 5 minutes. It was shown that a concentration of glucose in serum up to 400 mg per 100 ml did not influence the amount of formaldehyde found after oxidation of the extract aliquot with per-iodic acid. The FG includes glycerol and the half amount of l-monoglycerides (the latter give only 1 mole formaldehyde compared with glycerol, which gives 2 moles).

Determination of neutral-fat glycerol (NFG)

An aliquot of the extract was dried, and the residue saponified with 2.5 per cent potassium hydroxide in ethanol. The glycerol formed in this manner includes phospholipid glycerol, 'free glycerol' (FG), and neutral-fat glycerol. By subtracting the phospholid glycerol and the FG from the total glycerol the quantity of glycerol arising from neutral fat is obtained.

Correction for non-glycerol phospholipids has not been made.

Electrophoresis

Paper electrophoresis was performed in the apparatus of Dettker & Andurén (1954). Serum was diluted (2:1) with veronal buffer (pH 8.6, ionic strength 0.1). Of this, 30 mm^3, was applied to moistened Whatman No. 1 filter paper. Electrophoresis 18 hours. Strips were dried, and cut lengthwise. One part was stained with Amido Black (Grassman, Hanning & Knedel, 1951) for protein, and the other with Oil Red (Jenks, Durrum & Jetton, 1955) for lipids.

Histological specimen

Immediately after killing, the lower part of the right lung was removed and placed in formalin. After fixation frozen sections were cut and stained with scarlet red.

RESULTS

Neutral-fat glycerol (NFG). It was found that after trauma there is a pronounced increase of NFG in rabbit serum (Fig. 1). The mean value for group I, *i. e.* animals

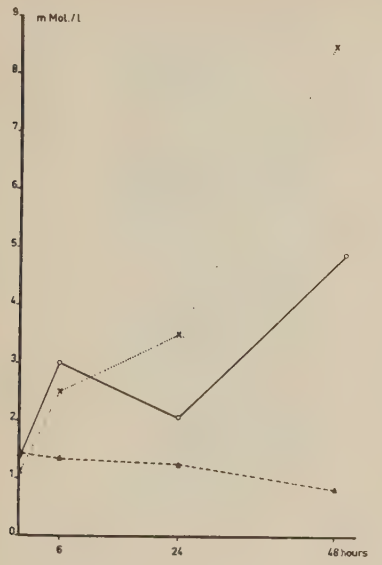

Fig. 1. Neutral fat glycerol.

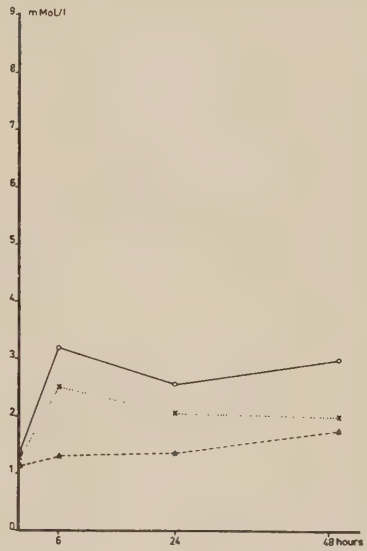

Fig. 2. 'Free glycerol'.

x ..... x Neutral fat glycerol (NFG) in rabbits exposed to tourniquet shock. Mean of 8 animals.

o ——— o NFG on rabbits exposed to tourniquet shock, subsequently injected with heparin. Mean of 8 animals.

▲ — — — ▲ Controls.

x ..... x 'Free glycerol' (FG) in rabbits exposed to tourniquet shock. Mean of 8 animals.

o ——— o FG in rabbits exposed to tourniquet shock and subsequently injected with heparin. Mean of 8 animals.

▲ — — — ▲ Controls.

xposed to tourniquet shock, before trauma as 1.0 mMol/l. After 24 hours the NFG ad increased to 3.4, and after 48 hours to 4 mMol/l. There was thus a roughly eight-old increase after 48 hours.

Animals exposed to tourniquet shock and bsequently injected with heparin (group , Fig. 1) did not show a corresponding crease in NFG. In group III the NFG mained fairly constant, but showed an significant fall at 48 hours.

'Free glycerol' (FG) in serum after uma. From Fig. 2 it is clear that the rum FG increases after trauma (see discussion). This increase was more pronounced in the group that received heparin. The control group showed a slight increase in FG, but no conclusions can be drawn from this.

Phospholipids. The serum phospholipids did not change to the same extent as the NFG. The results (Fig. 3) show an increase of about 4 times at 48 hours in rabbits exposed to tourniquet shock. The group that received heparin did not show a corresponding increase. The control group showed no change.

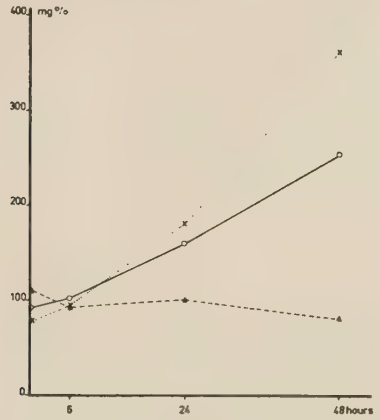

Fig. 3. Phospho-lipids.

x . . . . . x Mean of 8 animals exposed to tourniquet shock.

o ——— o Mean of 8 animals exposed to tourniquet shock and injected with heparin.

▲ — — — ▲ Controls.

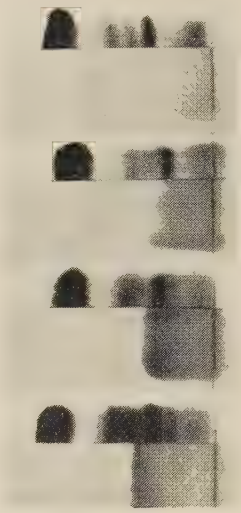

Fig. 4. Paper electrophoretic patterns of serum from rabbit exposed to tourniquet shock. Upper part of the strips protein staining; lower part lipid staining.

Fig. 5. Paper electrophoretic patterns of serum from rabbit 48 hours after it had been exposed to tourniquet shock and subsequently injected with heparin.

Electrophoresis. The dried paper strip was cut lengthwise. One part was stained for protein and the other for lipid. In this way it was possible to correlate the electrophoretic mobility of the lipid-staining fractions to that of the albumin and globulin fractions. Fig. 4 shows that it was possible to register two lipid fractions.

Before trauma (Fig. 4) the front of the slowest-moving fraction ('β-lipoproteins') lay between the γ- and β-globulin fractions. After trauma a change took place in this fraction. The mobility increased. After 24 hours the front corresponds to the β-globulin fraction, and after 48 hours to the α_2-globulin. Injection of heparin in the injured animal results in even more pronounced changes in the 'β-lipoproteins'. (Fig. 5). After 48 hours the front corresponds in the heparin-group to the α_1-globulin fraction. The control group does not show corresponding electrophoretic changes.

Paper electrophoresis and subsequent coloring of lipoproteins is associated with considerable error. No values for moving-distance or color-uptake of the lipoproteins will therefore be given, the qualitative changes mentioned above are only described.

Microscopy. As mentioned above, lipid-staining material has been found in the

pulmonary vessels of rabbits subjected to tourniquet shock (Johnson & Svanborg, 1956). It would be of interest to ascertain if treatment with heparin would reduce the amount of lipids present in the lung capillaries. With the technique used it has not been possible to draw any convincing conclusions from these experiments. By examining the slides, lipid-staining material was found in the vessels of both the heparin-treated and untreated groups, no marked difference being found. In the control group this change was not found.

DISCUSSION

It is not known in what way the depot fat is mobilized. It is possible that neutral fat is transferred directly to the blood, and that during transport in the blood it becomes hydrolyzed, and fatty acids are released. On the other hand it is possible that the neutral fat of the depots is hydrolyzed within the cells, the resulting fatty acids being taken up and transported by the blood.

From our results it is clear that an increased amount of neutral fat is present in rabbit serum after trauma. It can be excluded that the changes we have noticed in neutral fat can be referred to 'hemorrhagic lipemia' (Spitzer & Spitzer, 1955) or 'starvation lipemia'. The control group did not show any significant change in the neutral fat. The increased content of neutral fat in rabbit serum following trauma was thus due to the trauma *per se*.

Several investigations indicate that the neutral fat is transported by lipoproteins (Anfinsen, 1955). The increase in phospholipids found may perhaps be explained by an increase in the amount of lipoproteins, the chemical composition of which is unchanged, or by the inclusion of increased amounts of phosphatides in each lipo-protein molecule. It was observed that a lower concentration of neutral fat, as in the heparin-treated group, there was also a lower concentration of phosphatides. This might indicate a relationship between the amount of neutral fat and the concentration of phospholipids.

Our investigations showed that 'free glycerol' (glycerol and 1-monoglycerides) increased after trauma and that this increase was most pronounced in animals given heparin. Thus our findings suggest the possibility of increased lipolysis after trauma.

Increased hydrolysis of neutral fat results in increased amounts of non esterified fatty acids. Heparin-induced 'clearing' of lipemic serum gives such a large increase in non-esterified fatty acids that the binding-capacity of albumin is exceeded, the excess being bound to lipo-proteins. Non-esterified fatty acids give the lipo-proteins a higher net charge, resulting in increased mobility of the lipo-proteins in the electric field (Herbst, Lewer & Lyons, 1955; Laurell, 1955). Carlsson & Wadström (1956) have shown that the electrophoretic mobility of the lipo-proteins in paper electrophoresis was affected not only by the electric field but also by the adsorption of the lipo-proteins to the paper. They studied, by means of buffer flow experiments without any current, the adsorption of lipo-proteins to the paper at various levels of pH. At higher pH values they found adsorption to the paper to be less. This indicates lesser adsorption of lipo-proteins given a higher net charge.

Our investigations showed that trauma is followed by qualitative changes in the serum lipo-proteins. The 'β-lipo-proteins' increased in quantity, and showed increased electrophoretic mobility. The most probable explanation of the latter phenomenon is that fatty acids released by hydrolysis of neutral fat in blood and/or directly taken up by the blood from fat-depots become bound to lipo-proteins, and give them a higher net charge. This assumption is supported by the fact that injections of heparin in injured animals are followed by still more pronounced changes in the electrophoretic pattern of the lipo-proteins.

SUMMARY

1. Experimental tourniquet shock in rabbits resulted in a rise in the neutral fat content by about 8 times within 48 hours.

2. Following trauma the amount of 'free glycerol' (*i. e.* glycerol and 1-monoglycerides) increased. The significance of this observation is discussed.

3. Injection of heparin in injured animals results in lowering of the serum neutral fat and increase of the 'free glycerol' concentration, compared with animals not given heparin.

4. The content of phospholipids in serum increased about 4 times during the 48 hours following trauma. A possible relationship between the content of serum neutral fat in serum and the serum phospho-lipids is discussed.

5. Increased electrophoretic mobility of the 'β-lipo-proteins' was observed after tourniquet shock in rabbits.

6. Lipid-stained frozen sections of lung tissue show the presence of large quantites of lipid material in the capillaries. No marked differences was found between heparin-treated animals and animals not given heparin.

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A NOMOGRAPHIC SOLUTION FOR LUNG VOLUME DETERMINATIONS IN THE CLOSED SYSTEM HELIUM DILUTION METHOD

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(Received for publication June 7, 1956)

To facilitate the determination of the functional residual capacity (FRC) of the lungs in the closed system helium dilution method (Holmgren, 1954), a nomographic solution has been developed. The advantages of this technic are the speed and freedom from calculations which it provides. Fig. 1 is a reproduction of the nomogram developed for use in connection with our spirometer. Since the construction depends on the individual spirometer in use, a rather complete description of its derivation is presented in order that others using the closed system helium dilution method will be enabled to construct such a labor-saving device.

STEPS IN THE USE OF THE NOMOGRAM

Step 1. On scale *h* is set the height of the spirometer bell at the beginning of the study. This height in mm represents the volume of air-helium mixture which has been previously introduced into the empty spirometer bell. This point on the *h* scale is connected by a straight line to a point on the [He]n scale representing the helium concentration at the end of the study. Where this line crosses the diagonal reference line, a mark is made.

Step 2. Another straight line is passed from the point on the [He]n scale through a point on the [He]o scale corresponding to the helium concentration at the beginning of the study. The point where this line meets the vertical reference at the extreme left is marked. This point's level is led horizontally over to the [He]n scale and the [He]n scale marked accordingly.

Step 3. From the new point of the [He]n scale a straight line is drawn through the previously found intersection on the diagonal reference line and the line extended until it meets the FRC Scale. If the end-expiratory level at the end of the test has changed from that at the beginning, a suitable correction is added for each mm elevation or subtracted for each mm drop.

MATHEMATICAL DERIVATION OF THE NOMOGRAM

If one assumes that the amount of helium in the system is the same at the beginning as at the end of the study, the following equation holds true:

* With the Technical Assistance of P.-A. Hallberg and A. Åhbeck.

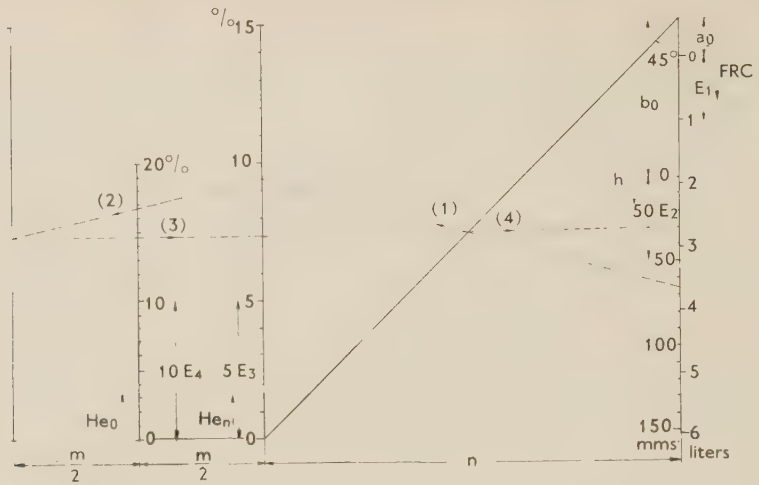

Fig. 1. Construction data adaptable to any given spirometer to provide a nomographic solution for functional residual capacity calculations. Suitable values for scale lengths, distances from one scale to another, modules, and points of origin are given. The broken line and the figures in parentheses show the steps taken in the use of the monogram. See text for details of construction and use.

$$[He]o \times V_{sp} = [He]n \times (V_{sp} + Z) \quad (1)$$

where [He]o and [He]n are the concentrations of helium in the spirometer at the beginning and the end of the study, respectively,

V_{sp} is the volume of the spirometer system including tubing, fan, *etc.*

and Z is the FRC plus the dead space volume (V_D^{app}) extending from the mouthpiece to the stop cock of the spirometer.

For the construction of the nomogram, the form of equation (1) hos been altered too:

$$\frac{V_{sp}}{[He]n} = \frac{Z}{[He]o - [He]n} \quad (2)$$

To simplify the use of the nomogram the spirometer bell volume (scale h) is graduated in mm spirometer bell height (kymographically recorded) and its zero point corresponds to V_{acc}, the volume of the accessory parts of the spirometer system (tubing, fan, *etc.*) The relationship between the spirometer system volume (V_{sp}) and height (h) may be expressed as follows:

$$V_{sp} = h \times K + V_{acc} \quad (3)$$

where K is the spirometer's volume factor in liters per mm excursion.

The FRC plus the apparatus dead space are corrected to ATPS conditions of the spirometer bell (about 20° C.) from BTPS by a factor of 1.1, giving the following relationship:

$$Z = \frac{FRC + V_D^{app}}{1.1} \quad (4)$$

Substituting the right hand members of equations (3) and (4) into equation (2), one obtains:

$$\frac{h \times K + V_{acc}}{[He]n} = \frac{FRC + V_D^{app}}{1.1([He]o - [He]n)}; \text{ or}$$

$$\frac{FRC + V_D^{app}}{1.1} = \frac{h \times K + V_{acc}}{[He]n}([He]o - [He]n)$$

The mathematical significance of the different steps in the use of the nomogram thus becomes:

Step 1. Calculation of the quotient $\frac{h \times K + V_{acc}}{[He]n}$. This is obtained from the diagonal line intersection.

Step 2. Calculation of the drop in helium concentration, $[He]o - [He]n$.

Step 3. Calculation of the product

$$\frac{h \times K + V_{acc}}{[He]n}([He]o - [He]n).$$

Reading the solution of this on the right hand scale gives the FRC if the volume of the system at end-expiratory level has remained constant. If this is not the case, equation (1) is utilized as follows:

$$[He]o \times V_{sp} = [He]n \times (V_{sp} + \Delta V + Z) \quad (6)$$

where $\Delta V = \Delta h \times K$ the change in the volume of the system in which Δh is the change in the spirometer bell's height.

Solving for Z in this equation yields:

$$Z = \frac{V_{sp}}{[He]n}([He]o - [He]n) - \Delta h \times K.$$

Since this represents the solution of Step 3 minus the change in the volume of the patient-spirometer system, it is evident that the FRC must be corrected by 1.1 times the spirometer factor for each mm rise or drop of the end-expiratory level.

CONSTRUCTION OF THE NOMOGRAM

If the spirometer is of the Knipping type with a volume-excursion factor (K) of approximately 0.02 liter/mm, an accessory volume (V_{acc}) of approximately 3 liters, and a dead space volume (V_D^{app}) of about 0.05 liter, by taking the *h* scale's module E_2 as unity, the following relationship holds true:

$$a_o = \frac{V_D^{app} + 0.1^*}{K \times 1.1} = \frac{0.05 + 0.1}{0.02 \times 1.1} = 6.8 \text{ mm.}$$

$$b_o = \frac{V_{acc}}{K} = \frac{3}{0.02} = 150 \text{ mm.}$$

$$E_1 = \frac{1}{K \times 1.1} = \frac{1}{0.02 \times 1.1} = 45.5$$

The FRC scale, if the upper extreme is taken as 6 liters, will measure about 270 mm. To approximate this length, the [He]n scale's module, E_3, can be taken as 20 mm, giving the scale a length of 300 mm. Since the diagonal reference line meets the FRC and [He]n scales at 45°, the distance *n* between them will also be approximately 300 mm.

The [He]o scale is placed midway between the [He]n scale and the vertical reference line; the module here will then be 10 mm (E_4). The distance *m* between the [He]n scale and the vertical reference line can be arbitrarily chosen to be about 160 mm; we have found this to provide good readability.

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* Because of a small but significant amount of helium absorption which takes place, we have corrected the FRC scale by a 0.1 liter shift downward (Birath & Swenson, 1956).

SUMMARY

A labor-saving nomographic method for the calculation of the lungs' functional residual capacity is described. Because such a method depends on the characteristics of the individual spirometer, a detailed mathematical derivation and directions for construction are also presented.

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A NOTE ON THE OCCURRENCE OF RIBOSURIA IN STATES OF MUSCULAR DYSTROPHY

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In 1949 Minot, Frank & Dziewiatkowski reported the occurrence in the urine from cases of progressive muscular dystrophy of a substance reducing Benedict's solution after prolonged boiling. The substance was found to be ribose, excreted in the urine as a phosphorous-containing complex.

A few years later Orr & Minot (1952) published a detailed method for the demonstration of ribose in urine. The method involved treatment of the acidified urine with Lloyd's reagent and subsequent hydrolysis and isolation of the sugar as the phenylosazone. Ribosuria could thus be demonstrated in 26 cases of different types of progressive muscular dystrophy, 6 cases of dystrophia myotonica, 5 cases of myotonia congenita and in 3 cases of amyotonia congenita. No ribose was found in the urine from cases with muscular atrophy of other origin, including a number of neurological conditions, except in one case of multiple myelomatosis.

As these results seemed to present a new laboratory test which might be of considerable value to the clinician and also offered new interesting aspects as to the mechanism of the muscular dystrophies we started to investigate the urine from similar and other cases.

PROCEDURE

The procedure of Orr & Minot with treatment with Lloyd's reagent was followed but then the samples were hydrolyzed at 100° C with sulphuric acid, final concentration about 2 N, for 1 hr., and after that partially desalted. The hydrolysates were finally analyzed by one-dimensional paper chromatography. The solvents used were butanol-acetic acid (Partridge, 1948) and lutidine-amyl-alcohol (Werner & Odin, 1949) and the papers were sprayed with aniline-phthalic acid solution (Partridge, 1949). This procedure is far more sensitive than the osazone formation test and allows more definite conclusions as to the kind of sugars present in mixtures and impure solutions.

RESULTS

10 cases representing different kinds of progressive musculatur dystrophy were examined. The presence of ribose in the urine was clearly established in 8 cases, traces of ribose was found in one case. In one case (pseudo-hypertrophic form which had remained "stationary for at least a decade) no ribose was found".

In 10 apparently normal, healthy medical students ribosuria could be demonstrated in 3 cases. All three of them denied having eaten fruits during the last two days preceding the test.

The presence of ribose in the urine was also demonstrated in one case of multiple

myelomatosis, in two cases of myeloic leukemia, in one case of lymphatic leukemia, in one case of prostate cancer with multiple skeletal metastases, in one case of pneumonia and in one case of rheumatic fever.

CONCLUSIONS

The presence in urine of hydrolysable ribose does not seem to be a very specific phenomenon provided sufficiently sensitive analytical methods are applied. The test thus seems to have a very limited value in the diagnosis of muscular dystrophy. There might be quantitative differences but as far as our experience goes this does not seem regularly to be the case. However, further studies must be carried out before this question can be definitely settled.

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TREATMENT OF LEAD POISONING WITH CALCIUM EDTA

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(Received for publication June 23, 1956)

Ethylene diamine tetra-acetic acid (EDTA) is a compound capable of forming stable chelating agents with multivalent metals, such as lead. The resultant lead chelate is rapidly eliminated by the body *via* the kidneys. The lead ion will replace the calcium ion in calcium EDTA. Hence this compound has been administered in cases of lead poisoning for the purpose of eliminating the toxic lead ion from the body. In 1952, Rubin, Gignac & Popovici tried it successfully in experiments on lead-intoxicated rabbits, and in the same year the first reports were published on successful calcium EDTA therapy in acute or chronic lead poisoning in human beings (Bessman, Reid & Rubin one case; Belknap 3 cases). Subsequently the favorable effect of calcium EDTA in this condition has been confirmed by several writers (Sidbury, Bynum & Fetz (1953) 7 cases; Hardy, Elkins, Ruotolo, Quinby & Baker (1954) 3 cases; Cotter (1954) 4 cases; Eilersen, Astrup, Fallentin & Frost (1955) 4 cases; Mellemgård & Sørensen (1955) one case; Glømme & Swensson (1956) 2 cases).

MATERIAL AND METHODS

Five patients were treated with intravenous infusions of calcium EDTA in glucose. Two of them had exhibited acute symptoms of lead poisoning. For this reason their fellow workmen in the storage battery factory where they were employed were examined, and thus the other three patients were detected. The latter showed mild subjective symptoms and definite objective signs of lead poisoning. All these patients had been handling lead plates in their work, and had probably become intoxicated by inhalation of the lead oxide powder covering the surface of such plates. The symptoms are summarized in Table I.

The therapy consisted in daily infusions of 2.5 g of calcium EDTA in 1000 cc of a 5 per cent glucose solution over a two or three hour period for ten days. The patients showing severer signs of

Table I. *Symptoms of 5 patients suffering from lead poisoning.*

Case	J. No.	Fatigue	Nausea	Abdominal pain	Anemia	Basophilic stippling of red cells	Complications
1	469/56	+	+	+	+	+	—
2	917/56	+	+	+	+	+	—
3	528/56	+	+	+	+	+	—
4	592/56	+	—	—	—	+	—
5	676/56	—	—	—	—	±	—

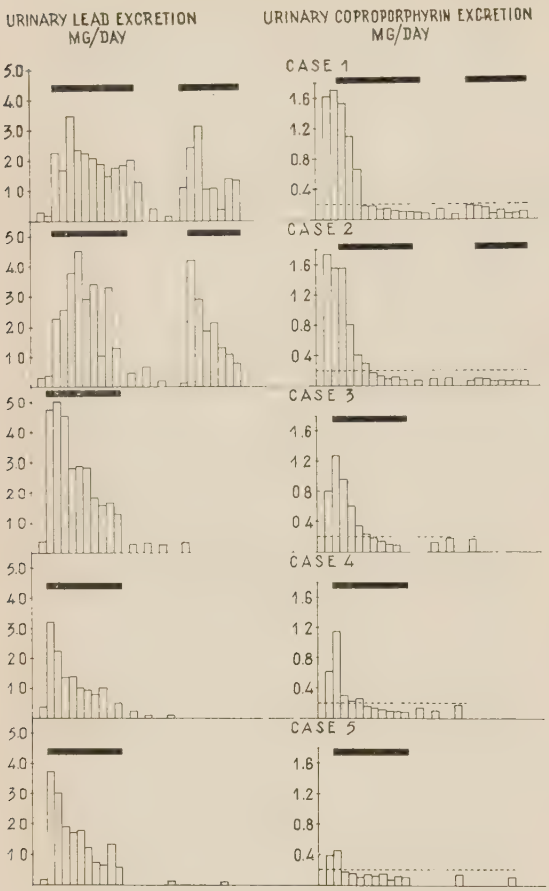

Fig. 1. Block lines: EDTA-medication.

poisoning were given two series of infusion with an interval of one week, those with mild signs one series as suggested by Lund in 1955.

Urinary coproporphyrin was measured daily from samples collected in dark flasks containing 5 g of Na_2CO_3 as described in 1951 by Schwartz, Zieve & Watson (Fig. 1). The coproporphyrin content of the daily output of urine was estimated photometrically with a Unicam SP. 600 spectrophotometer. Extraction was performed by the method of Schwartz *et al.*, though with the modification that the volume of urine was 10 ml. The spectrophotometric determination and the calculation of the results were performed by the method described by Askevold (1951) modified on the basis of With's (1955) investigations. Levels of under 200 µg daily were regarded as normal.

The daily *urinary lead excretion* was measured by the method described by Bessman & Layne in 1955 (Fig. 1).

RESULTS

In all five cases the calcium EDTA therapy had a favorable effect on the symptoms of lead poisoning. The nausea, crampy abdominal pain and fatigue disappeared, anemia — if present — was rapidly improved, and after therapy no basophilic stippling of the red cells was observable. The urinary coproporphyrin returned to the normal level within one or two days, and the daily excretion of lead increased manifold during treatment. None of the patients reported any untoward symptoms of the therapy, and the results of subsequent physical examination were within normal limits.

DISCUSSION

The present results confirm previous experiences of the favorable effect of calcium EDTA therapy in lead poisoning. It seems reasonable to assume that it is the circulating lead, and possibly — in view of the rapid return of coproporphyrin to the normal level — the lead stored in the liver, that is rapidly eliminated in this way, whilst the lead stored in the bones requires a repetition of the treatment for good results. It is thus acute cases of lead poisoning, and acute episodes of chronic cases, that respond most readily to this therapy, as has also been emphasized by other writers.

Recently a toxic effect of EDTA on the kidneys has been reported (Dudley, Ritchie, Schilling & Baker, 1955; Foreman, Finnegan & Lushbaugh, 1956 and Johansson &

Östling, 1956), injury to the renal tubules having been observed both in patients and in experimental animals. In the present cases no pathological findings were made in the urinary sediment after discontinuing treatment. During therapy, urinalysis was not performed with this object in view. The absence of such untoward effects is probably preferable to the moderate daily dose, 2.5 g, which is > 3.5 g less than the daily dose regarded as critical. Nonetheless, these unfavorable reports seem to constitute a contraindication to prophylactic treatment with calcium EDTA by mouth over any considerable length of time for subjects exposed to lead in their work.

SUMMARY

Five patients with chronic lead poisoning were treated with 2.5 g of calcium EDTA in 1000 cc of a 5 per cent glucose solution administered daily as intravenous infusions over two or three hours for ten days. No untoward effects of the therapy were observed, and the results were favorable in all cases. The daily excretions of lead and of coproporphyrin were determined, and the results exemplify the rapid and favorable effect of calcium EDTA on the excretion of the circulating lead.

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A RAPID AND EXTREMELY SENSITIVE TEST FOR METHANOL IN BLOOD AND BIOLOGICAL MATERIAL

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(Received for publication August 26, 1956)

In cases of acute alcohol intoxication the question often arises as to whether methyl or ethyl alcohol is involved. There is an obvious need for a rapid, sensitive, and specific method for the detection of methanol which can be used in hospital laboratories with limited chemical facilities. The new method meets these requirements.

METHOD

Principle

Methanol is oxidized to formaldehyde which is detected by its reaction with 2.7-naphthalenediol (Eegriwe, 1932). The oxidation is carried out under controlled conditions to prevent further oxidation to formic acid which does not react with the naphthol reagent.

In blood and biological material, several compounds besides methanol give formaldehyde on oxidation. Methanol is, therefore, isolated from the sample by distillation in a simple microdistillation apparatus (Fig. A and B).

Reagents

Conc. H_2SO_4, p.a.

Approx. 0.1 N $KMnO_4$

2.7-naphthalenediol solution prepared in the following way: 200 mg 2.7-naphthalenediol and 1.0 gm of sodium sulfite (anhydrous) dissolved in 100 ml of water. Sodium sulfite prevents oxidation of the reagent and reduces an excess of permanganate which is used in the test. The reagent keeps well in the refrigerator and will remain stable at room temperature for weeks without loss of reactivity.

Procedure

Take 5 ml of blood (with or without anticoagulant) and add 3—4 ml of water in the distillation flask.

If carbon dioxide as dry ice is available, model A is used and a mixture of acetone and dry ice placed in the cooler. The cooler is secured at the neck of the distillation flask by a perforated rubber stopper. The apparatus is transferred to a boiling water bath. A mixture of water and methanol vapors condense and freeze on the stem of the cooler. After two minutes the apparatus is removed from the bath, care being taken that none of the condensate is lost, the remaining cooling fluid poured out, and the stem placed in a test tube and allowed to drain. If a little hot water is poured into the cooler, the thawing is completed in a few seconds. The stem is rinsed with about 1.0 ml of water from a Pasteur pipette.

If dry ice is not available, model B is used. After the sample has been transferred to the distillation flask, the cooler is placed with the tip of the stem just above the outlet of the collector. Ice water and ice are poured into the cooler and the apparatus transferred to a shallow, boiling water bath. Distillation is carried out until ten drops have fallen from the tip of the cooler (about 2—3 minutes). The apparatus is removed, the cooler taken off, and the tube going from the distillation flask to the receptacle rinsed with 0.5 ml water.

The further procedure is the same in both cases. The test tube which now contains about 1 ml is placed in ice water, 0.1 ml of 0.1 N $KMNO_4$ added and then followed

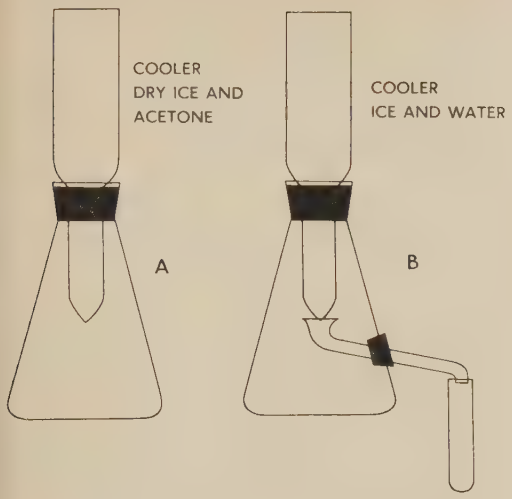

Fig. 1. Apparatus for microdistillation.

carefully by 0.5 ml concentrated sulfuric acid. The contents are mixed thoroughly. After two minutes, 1 ml 2,7-naphthalenediol reagent is added, and after mixing followed by 2 ml concentrated sulfuric acid and mixed again. The sample is removed from the ice water and placed on a boiling water bath for five minutes. A reddish-violet color develops in presence of methanol. A water blank should always be run simultaneously with the reagents. The blank should have a very faint yellowish or only a trace of reddish color. Any increased intensity above the blank denotes methanol.

RESULTS

Sensitivity: By using 5 ml of sample, a distinct color is produced by methanol in a concentration of 2.5 parts in one million.

Specificity: Formaldehyde and any substance which forms formaldehyde upon oxidation will give the reaction. Under the conditions given, no other volatile substance in the blood has been found to react. Ethyl alcohol, ethyl ether, beta-oxybutyric acid and acetone do not interfere.

SUMMARY

A rapid and sensitive test for methanol suitable for use in hospital laboratories is described. Methanol is distilled off by a simple microdistillation apparatus and after oxidation to formaldehyde is detected by a color reaction with 2,7-naphthalenediol.

Under the conditions given, no other volatile interfering substances, present in blood, have been found. As little as 2.5 parts in a million can be detected.

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STUDIES IN NORMAL ADULTS FOR VARIATION IN ZINC TURBIDITY TEST WITH SEX AND AGE

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Some investigations of the range of normal variation of Kunkel's (1947) zinc turbidity test are available. Löwgren (1949), for example, whose material consisted of 104 apparently healthy persons, mainly men (96 males and 8 females) aged 10 to 49, found a mean value of 5.65 ± 0.18 with a standard deviation of ± 1.88 units/100 ml serum. He found a normal range of 2—10 units/100ml, as against a range of 2 to 8 units/100 ml given by Kunkel (1947).

A search of the literature failed to reveal any investigation of the zinc turbidity test for variation with sex and age. It was therefore considered desirable to study this problem in a series of apparently healthy adults.

MATERIAL

The material consisted of 117 adults (65 males and 52 females) of various ages. All of the subjects claimed that they felt well and inquiry into their family history revealed nothing of interest. In none had a thorough clinical examination revealed symptoms or signs of disease. The clinical examination comprised general physical examination, measurements of the blood pressure, electrocardiographic examination, fluoroscopy of the chest, urinalysis of morning specimens for the occurrence of proteins, reducing substance and urobilinogen, examinations of the blood including determination of the erythrocyte sedimentation rate and icterus index (a value of 7 was taken as the upper normal limit). Only macroscopically clear sera were accepted.

All samples were presumably collected in the post-absorbtive state, all of the donors having stated that they had neither eaten nor drunk anything for the previous 10 to 12 hours. None of the females was pregnant and their samples were collected intermenstrually, and never just before, during or just after menstruation. The samples were free from hemolysis. All measurements were made in duplicate, and all the results are averages of these duplicate analyses.

METHOD

The test was performed according to Löwgren (1949). The extinction values (read in a Beckman spectrophotometer model B at a wavelength of 650 mμ in a 1 cm cuvette) are multiplied by 23, the product giving the result of the zinc turbidity test in so called units/100 ml serum.

In order to obtain an idea of the accuracy of the method, use was made of the duplicate determinations of the extinction values and the standard error of a single determination calculated according to the formula $\pm\sqrt{\frac{\Sigma d^2}{2N}}$. This gave a value of 0.01, which corresponds to ± 0.23 units/100 ml serum. No difference was found between the sexes.

RESULTS

The results and statistical analysis of the data obtained are given in Tables I and II.

DISCUSSION

Analysis of the distribution shows that the majority of the observations fell within a range of 1.0 to 4.9 units/100 ml serum. A few isolated high values were recorded. These sometimes exerted a relatively strong influence on the means in the individual age classes. Neither in the material as a whole nor in the individual age classes was the test found to vary with sex. The differences found between the various age classes were not greater than what could be ascribed to chance and were due mainly to the occurence of isolated relatively high values.

Table I. *Distribution of the zinc turbidity test for men and women of different ages.*

Units/100 ml serum	Age in years										Total
	20—29		30—39		40—49		50—59		60—		
	m	f	m	f	m	f	m	f	m	f	
1.0—1.9	2	2	1	0	4	0	1	2	1	1	14
2.0—2.9	4	4	4	0	3	2	3	4	4	0	28
3.0—3.9	4	1	2	3	5	4	3	2	4	4	32
4.0—4.9	0	5	0	1	1	1	3	1	2	0	14
5.0—5.9	0	2	2	3	1	1	1	1	4	1	16
6.0—6.9	0	0	1	1	0	2	0	0	3	0	7
7.0—7.9	0	1	0	1	0	2	0	0	1	0	5
8.0—8.9	0	0	0	0	0	0	0	0	0	0	0
9.0—9.9	0	0	1	0	0	0	0	0	0	0	1
Total	10	15	11	9	14	12	11	10	19	6	117

Table II. *Results of a statistical treatment of the values given in Table I.*

Age in years	Mean values			Diff.
	m	f	m+f	
20—29	2.71	3.84	3.39	+1.13
30—39	4.06	5.00	4.49	+0.94
40—49	2.80	4.85	3.75	+2.05
50—59	3.57	3.02	3.31	—0.55
60—	4.29	3.37	4.07	—0.92
Total	3.57	4.06	3.79	+0.49
Error of the mean	±0.21	±0.23	±0.15	
S.D.	±1.63	±1.70	±1.66	

SUMMARY

An investigation of the zinc turbidity test of normal adults for any variation with sex and age in a material, in which all of the 117 subjects (65 males and 52 females) claimed that they felt well and in whom careful clinical examination revealed no symptoms or signs of disease.

The zinc turbidity test was not found to vary with sex or age.

The overall mean value was found to be 3.79 with a mean error of ± 0.15. St. Dev. ± 1.66 units/100 ml serum. The limits lay between 1.2 and 9.2 units/100 ml serum.

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AN INEXPENSIVE COOLING SYSTEM FOR INCUBATION TYPE WATER BATHS

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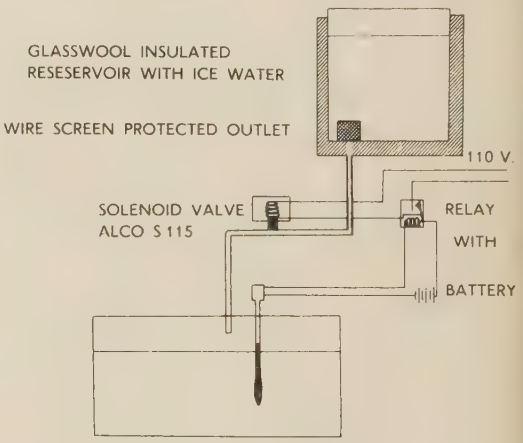
Whenever it is desirable to work below room temperature, a cooling system has to be used in connection with incubators. Several commercial units are available, but they are rather expensive.

The system described here will provide cooling for a medium sized incubator at a reasonable cost. A Dubnoff metabolic incubator was converted to work at any temperature between 0° C and room temperature. Temperature could be controlled to ± 0.1° C.

As it will be seen from the diagram, ice water, or any cooling mixture, is fed from a reservoir through a solenoid operated valve and into the bath which has an overflow outlet. The solenoid is operated at 110[1] v. a. c. and regulated through a contact thermometer with a suitable relay.

A 20 liter reservoir is used. The container is insulated with glasswool and corrugated cardboard. The outlet from the container is protected by a wirescreen to avoid clogging of the solenoid valve by ice and particles. Water together with ice, or still better carbon dioxide as dry ice, is used as cooling medium. Two kilograms give several hours of cooling.



INCUBATOR WITH CONTACT THERMOMETER

[1] Solenid valves are also available for 220 v.

Letters to the Editors

DETERMINATION OF HEXOSAMINES IN KIDNEY TISSUES

Boas (1953) has demonstrated the presence of chromogens which interfere with the color produced in his modification of the hexosamine method of Elson & Morgan (1933), when applied to animal tissues. The hydrolysates of almost all rat tissues tested produced interfering colors. The color produced was red with absorption maxima at 530 mμ and 560 to 570 mμ. By examination of absorption spectra, Boas found that sugars including glucose, mannose, fructose, galactose, glucuronic acid, ascorbic acid, sedoheptulose, ribose, DL-glyceraldehyde and dihydroxyacetone produced the same type of interference with absorption maxima at 530 mμ and 560 to 570 mμ. Most of the amino acids studied did not interfere with the hexosamine determination, whereas a reaction product formed from sugars and amino acids was chromogenic. This is also shown by Immers & Vasseur (1952). Hexosamines, however, were the only substances shown to produce an absorption spectrum with *one* peak at 530 mμ. Boas therefore emphasizes the importance of examining the absorption spectra in establishing the presence or absence of interfering chromogens in biological tissues.

Boas presents a quantitative ion exchange method with Dowex 50 for the separation of hexosamines from the chromogens. Recovery experiments with glucosamine · HCl gave very good results.

If the Elson & Morgan reaction could be applied without the ion exchange separation, however, this would be timesaving. The author has published a modification of Elson & Morgan's method, (Rosenlund, 1956), and it has been shown that glucose-lysine · HCl does not interfere with the color produced by this modified method.

As a part of an investigation concerning diabetic renopathy, this method has been applied to kidney tissues from rat and from normal and diabetic humans.

The kidney tissues were cut in pieces, washed in distilled water and frozen to — 80° C. The frozen material was immediately pulverized and freeze-dried. Ten to twenty milligrams of the material was hydrolyzed with 2 ml 2N HCl for 13 hours and the hexosamine content determined in the hydrolysate after neutralization. Absorption spectra of the colors produced by hydrolysate from rat kidney and by a solution of pure glucosamine · HCl are presented in Fig. 1. The courses of the curves are identical and there is no peak at 560 to 570 mμ on the curve representing rat kidney hexosamine. If, as postulated by Boas (1953), the absorption spectrum of the red color produced by hexosamines is specific, this means that the author's hexosamine method may be applied to rat kidney tissue hydrolysate without any preceding resin separation.

Boas' hexosamine method was also applied to the same kidney tissue hydrolysate without preceding separation. The absorption spectrum of the color produced showed only one peak at 530 mμ (Curve 1, Fig. 2) and the findings of Boas were thus not confirmed. If, however, the acetyl acetone concentration was considerably raised, *a blank* produced a color with an absorption spectrum which showed 2 peaks, at 530 mμ and

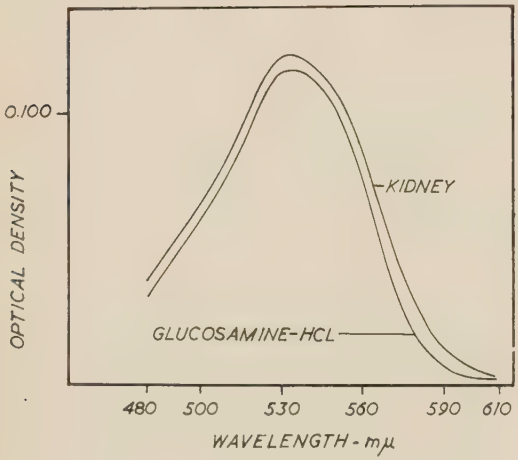


Fig. 1. Absorption spectra of the colors produced by hydrolysate from rat kidney and from a solution of pure glucosamine. HCl. Author's modification of Elson & Morgan's method. (Hilger, Uvispek spectrophotometer.)

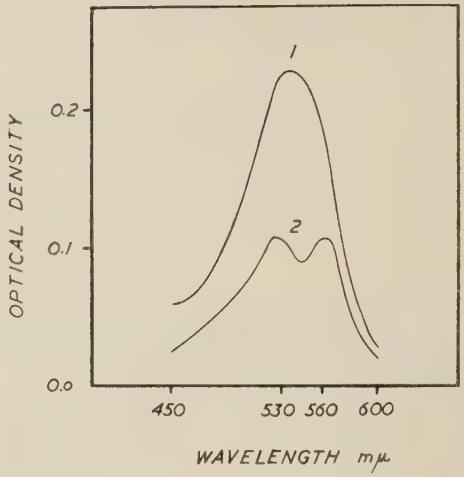


Fig. 2. Absorption spectra of color products in the hexosamine method of Boas. Curve 1: Rat kidney hydrolysate. Curve 2: A blank prepared with 8 per cent acetyl acetone in the acetyl acetone — sodium carbonate reagent, read against a blank prepared after Boas with 2 per cent acetyl acetone in the reagent.

at 560 to 570 mμ when read against a blank containing the usual amount of acetyl acetone (Curve 2, Fig. 2).

The condensation of acetyl acetone and Ehrlich's reagent may thus *alone* form a colored product which produces an absorption spectrum with peaks at 530 mμ and 560 to 570 mμ. The author has observed that impure acetyl acetone, in concentrations far lower than employed in these experiments, may produce a similar red color in the blank. This indicates that some split products from the acetyl acetone probably are responsible for the formation of this "false" color. Such split products may be formed in the reagent after a certain period of time, or/and produced during the acetylation on some occasions. It is therefore important that only acetyl acetone of highest purity should be employed in the hexosamine methods.

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Rikshospitalet,
September 6, 1956.
Oslo, Norway.

There is a striking similarity between the absorption spectra of color products from non resin separated tissue hydrolysate as found by Boas, and from a blank with high acetyl acetone concentration as shown by the author. The findings of Boas may therefore have some connection with this phenomenon of "auto-condensation" with the subsequent development of interfering colors.

Further investigations concerning the specificity of the author's hexosamine method when applied to other biological tissues are in progress.

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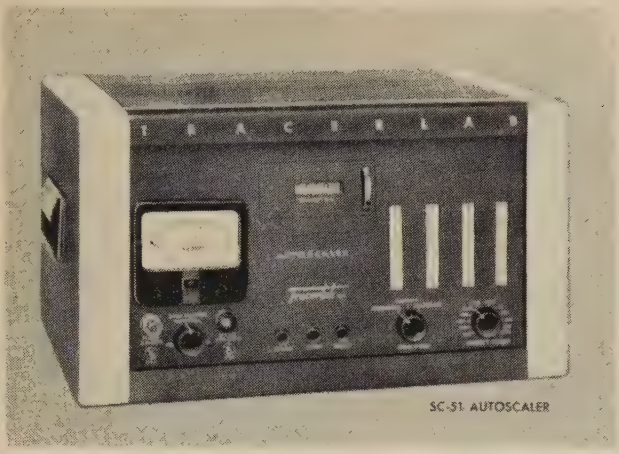

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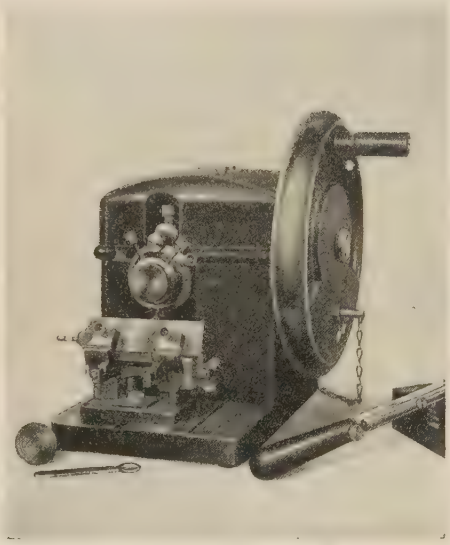

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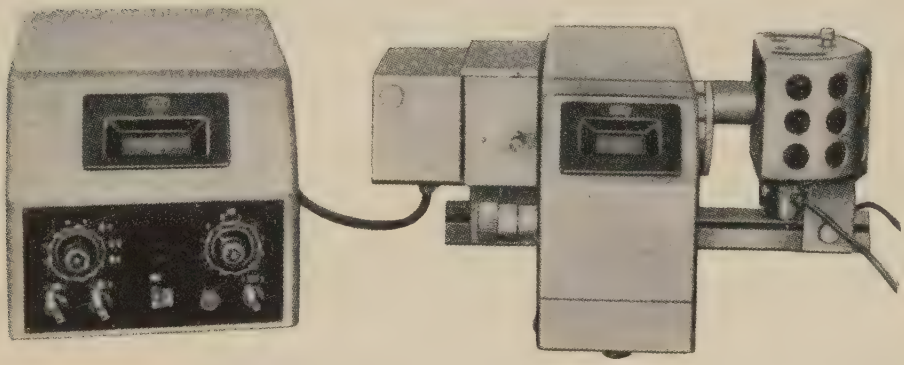

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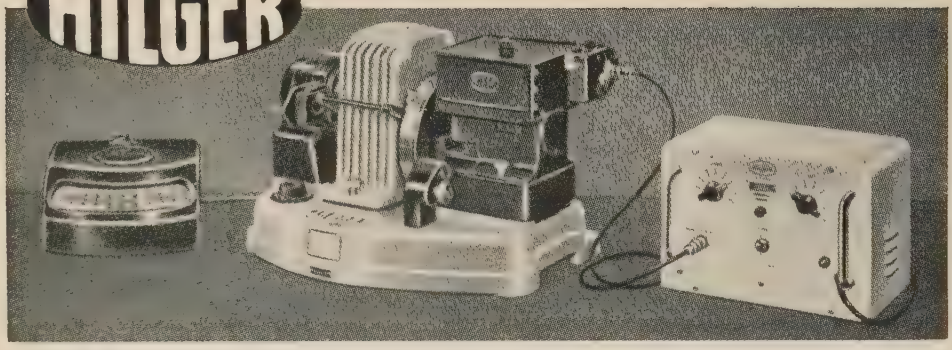

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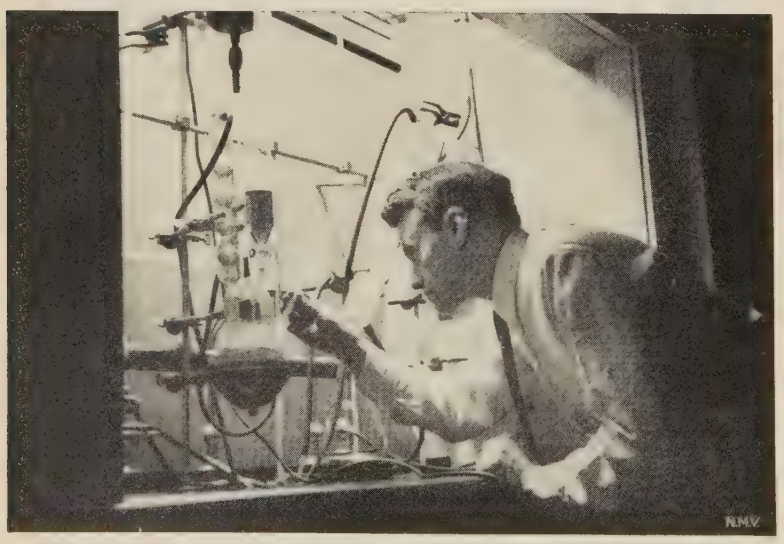

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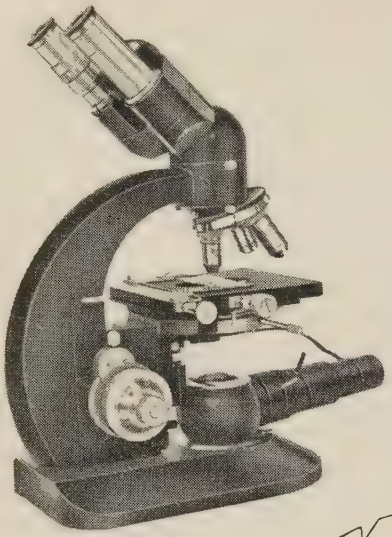

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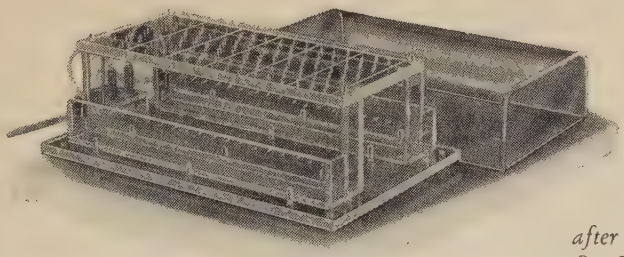

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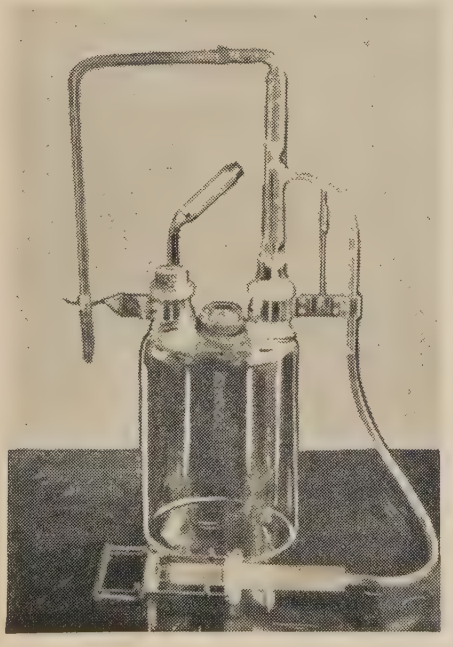

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